Chronic social stress and the circadian system

Effects on the central clock and peripheral liver oscillator

The project presented in this thesis was a collaboration between the University of Groningen (RUG), The Netherlands, and Universidade Federal de São Paulo (UNIFESP), Brazil. The research was carried out in the Chronobiology group at the Groningen Institute for Evolutionary Life Sciences (GELIFES - RUG) and the Group of Studies on the Neurobiology of Stress and its Disorders at the Department of Psychobiology (UNIFESP). The study was supported by a Bernoulli scholarship from the Faculty of Science and Engineering at the RUG, and a scholarship from Coordenação de Aperfeicoamento de Pessoal de Nível Superior (CAPES), Brazil.

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Chapter 1

General Introduction

1. General Introduction

Daily rhythms in behavior and physiology such as rhythms in rest and activity, feeding and body temperature, can be observed across the animal kingdom. Most of these rhythms are generated and controlled by an endogenous system of oscillators or clocks that reside in tissues throughout the body (Dibner et al, 2010). The endogenous oscillators are in fact present at the cellular level and are based on rhythms in expression patterns of so-called clock genes and maintained by molecular transcriptional and translational feedback loops (see figure 1, Mohawk et al, 2012, Bollinger and Schibler, 2014).

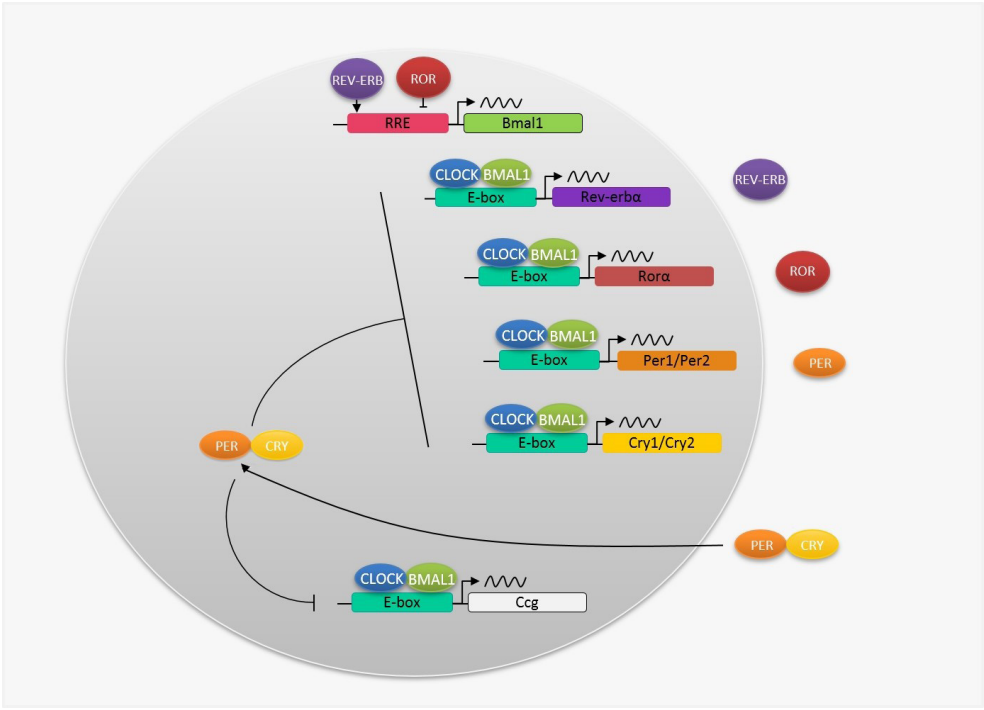

Figure 1. Molecular clock machinery, autoregulated by feedback loops. The transcript factors CLOCK and BMAL1 activate the transcription of Per1, Per2, Cry1, Cry2, Rev-erbα and Rev-erbβ genes. In the main feedback loop, PER and CRY proteins form complexes that repress the activity of CLOCK and BMAL1. In a second feedback loop, REV and ROR compete for binding to Bmal1 promoter region and inhibit or activate transcription. (Mohawk et al., 2012, Bollinger and Schibler, 2014) Adapted from Mohawk et al., (2012).

Central to the endogenous circadian clock system is a master clock, which in mammals is located in the suprachiasmatic nuclei (SCN) of the hypothalamus (Dibner et al., 2010, Mohawk et al., 2012, Ralph et al., 1990). This master clock directly drives rhythms in many functions by itself (Moore and Eichler, 1972, Stephan and Zucker, 1972) but also coordinates clocks or oscillators in other tissues and synchronize all the endogenous rhythms

to the outside world (Dibner et al., 2010, Mohawk et al., 2012). The SCN receives photic information from the retina through the retino-hypothalamic tract (Moore and Lenn, 1972) and thereby synchronizes the circadian system to the external light-dark cycle, allowing the organism to prepare and set metabolic processes to the optimal time of the day (Daan 1981; Moore-Ede 1986). Although temperature, food and other environmental signals can influence the circadian system, the most important cue to mammalian species is the light-dark cycle (Pittendrigh, 1981, Buhr et. al, 2010).

Shiftwork, jet-lag, social jet-lag or aberrant eating patterns results in disruption of synchrony among endogenous circadian rhythms and the environmental light-dark cycle, which leads to sleep disturbance, fatigue, reduced attention and performance. In the long run, chronic circadian disruption may have a serious impact on health and has been linked to increased sensitivity to a wide variety of diseases. For instance, prolonged exposure to shiftwork is associated with an increased incidence of certain forms of cancer and metabolic syndrome (Davis et al., 2001, Haus and Smolensky, 2006, Knutsson and Kempe, 2014, Tucker et al., 2012).

Although the circadian system is well adapted to respond to predictable cues in the environment, we asked ourselves whether it is susceptible to unpredictable and uncontrollable situations that trigger a stress response in the body. Conditions of chronic stress are associated to development of psychiatric disorders, which show strong alterations in daily rhythms in behavior and physiology (e.g., disrupted sleep-wake rhythm, disturbed rhythms in food intake and metabolism, as well as disturbed neuroendocrine rhythms) (Landgraf et al., 2014). One might argue that stress impacts the circadian organization and that rhythm disruption could be one of the important mechanisms underlying the stress-related diseases such as cardiovascular diseases and psychiatric disorders. In this thesis, we sought to answer the question whether stress can influence the circadian system and through which mechanism.

2. Stress does not appear to affect the master clock in the SCN

The early studies by Curt Richter in the 1960's already implied that stress does not seem to have major effects on the endogenous clock in the hypothalamus controlling the circadian rhythm in activity. Severe forms of stress, such as forced swimming, restraint and electrical shocks were applied, sometimes for several days in a row, and although overall activity level was often strongly suppresses, after the end of stress exposure, the activity would gradually normalize and more or less restart at the predicted time, indicating that the period and phase of the master clock had not been affected (Richter, 1967).

Later studies, with different approaches and more detailed analyses confirm the earlier findings. Studies in our laboratory have specifically addressed the question of whether changes in activity and body temperature rhythm that result from uncontrollable social stress are a consequence of changes in the endogenous circadian timing system. Rats

were exposed to acute social defeat stress in the first half of the activity phase (Meerlo et al. 1997) or in the middle of the resting phase (Meerlo and Daan 1998), and in neither one of these studies there was an effect on the phase or the period of the free running rhythms. Although the output rhythm may become masked by stress-induced disturbances elsewhere in the body, the central pacemaker in the SCN seems to be unaffected.

These findings on exposure to social defeat in rodents are supported by the vast majority of available data collected with a variety of acute stress models and most reports on effects of more chronic forms of stress. However, data on chronic stress are less consistent and not always easy to interpret. For example, a recent study on chronic social defeat in mice has reported that defeated mice lost the first activity bout during the subjective dark phase and display shorter activity and body temperature periods compared to control mice during the 14 days after the stress protocol. (Bartlang et al., 2015). However, the effects on rhythm period were small (a shortening of 0.2h) and were only observed in C57BL/6N mice but not in the C57BL/6J strain.

Manipulations of hormones of the hypothalamic–pituitary–adrenal axis, one of the main neuroendocrine stress-responsive systems, also suggest that the master clock is not easily disturbed by stress. While adrenalectomy (ADX) decreases the level of wheel running activity, it does not appear to affect the timing of activity, as this activity remains concentrated in the dark phase Moberg and Clark (1976). Moreover, administration of dexamethasone (DEX), a synthetic glucocorticoid (class of hormones produced by the HPA axis), to ADX rats, in the beginning of the light or the dark phase, increases running wheel activity after injection, but does not change the phase of the circadian rhythmicity (Moberg and Clark, 1976). Neither continuous release nor a 6 h cortisol (a glucocorticoid hormone) daily pulses affect the period or phase of the free-running activity rhythm in ADXed hamsters (Albers et al., 1985).

Together, most data on acute stress and glucocorticoid administration are clear and show no effect on phase and period of SCN controlled rhythms. Results from studies on chronic stress seem to support this conclusion but the data are less consistent and not always easy to interpret. Perhaps chronic stress in the long run may result in small cumulative effects that go undetected with acute stress, which is one of the issues addressed in this research project. For a more detailed overview of effects of stress and stress hormones on circadian function, see chapter 2.

3. Stress may affect peripheral oscillators

Another important remaining question is whether stress or stress hormones can affect other oscillators in various tissues throughout the body, which are normally under control of the central clock in the SCN (Balsalobre et al., 2000, Dibner et al., 2010).

In a study by Razzoli and colleagues (2014), chronic social stress did not affect the locomotor activity rhythm, but induced phase advance of the PER2 clock protein peak in the

adrenal and pituitary glands. Similarly, restraint stress at ZT4-6 for 3 days/week, for 4 weeks, induces PER2 rhythm phase advance in peripheral tissues (kidney, liver and submandibular gland) (Tahara et al., 2015). However, acute stress appears to have transient effects on clock gene expression, since restraint stress for 1 h enhances Per1 mRNA levels in mouse liver, heart, lung and stomach, without any alteration in other clock genes (Yamamoto et al., 2005).

A study with DEXA injections showed that this synthetic glucocorticoid altered rhythmic clock gene expression in cell cultures from liver, kidney, and heart tissue, while it did not affect gene expression in neurons of the SCN (Balsalobre et al., 2000). This result goes in line with the finding that the adult SCN does not present glucocorticoid receptors (Rosenfeld et al., 1988).

It may be that certain stressors affect peripheral oscillators, perhaps temporarily disturbing their control by the SCN, leading to a state of internal desynchronization. Such internal desynchronization might in part be responsible for the disturbances in overt rhythmicity in body temperature and locomotor activity seen after social defeat stress.

4. Outline of the studies

In this thesis we addressed two main questions. The first on was whether the circadian master clock in the SCN, that appears to be well-protected against acute stress, is perhaps affected by more chronic stress. The second question was whether circadian oscillators other than the SCN might be sensitive to stress and stress hormones (figure 2).

In **Chapter 2**, we provide a detailed review of previous literature on the effects of stress on the circadian system. While it is generally accepted that light is the main cues that synchronizes the mammalian circadian system to 24h environmental cycles. Nevertheless, previous work has indicated that the circadian system may be sensitive to a variety of so-called non-photic inputs (Mrosovsky, 1996) and the question we addressed is whether stress signals or stress hormones, one way or another might, have access to the endogenous circadian system as well. The chapter also provides a detailed overview of the various models and approaches that are used to address the question of stress effects and we discussed the importance and limitations of these models and approaches.

In **Chapter 3**, we present our experiments in mice that were aimed at investigating the effects of chronic social defeat stress on the SCN-controlled circadian rhythm in locomotor activity. Mice were maintained under constant, free-running conditions throughout the experiment (constant dim red light) and different groups of animals were exposed to repeated defeat for 10 consecutive days, either during their activity phase or during their resting phase. Using activity onset as phase marker of the endogenous free-running activity rhythm, we assessed whether chronic stress would result in phases shifts or changes in the circadian period. Based on most of the available literature reviewed in chapter 2, we expected that repeated social defeat stress would suppress activity levels but

would not affect the phase and period of the rhythm.

In **chapter 4**, we used *in vivo* and *in vitro* measures to investigate the effects of repeated social defeat stress on the circadian rhythms. The observations on the effects of chronic social defeat on the activity rhythm during stress exposure in Chapter 3 were replicated in transgenic PER2::LUC mice, which allowed us to assess PER2 protein abundance through bioluminescence recording of the fused protein fusion construct. Those animals were euthanized after the 10-day stress period, and liver and SNC tissue were collected to assess if the expression of the clock protein PER2 was affected in the master clock and/or the peripheral oscillator in the liver. Based on the available literature, we expected that repeated social defeat stress would neither affect PER2 expression in the SCN nor its output in terms of phase and period of the activity rhythm. However, bases on a number of recent studies, we anticipated that social defeat stress might affect the liver rhythm in PER2 expression.

Chapter 5 was an initial study to assess the potential mechanism by which stress could signal to peripheral oscillators and perhaps, to the master clock. The stress hormone corticosterone was applied directly in the culture media of liver and SCN tissues and PER2 expression rhythm was assessed. We expected to find results similar to those of chronic social defeat stress.

Considering the hypothesis that chronic social stress may lead to internal desynchronization, and that could play a role on psychiatric disorders, such as depression and anxiety, we evaluated the effects of chronic social stress on depressive- and anxiety-like behavior in adult male mice in **Chapter 6**.

Finally, in **chapter 7** we provide a summary of the main findings and a discussion of the results in relation to the literature.

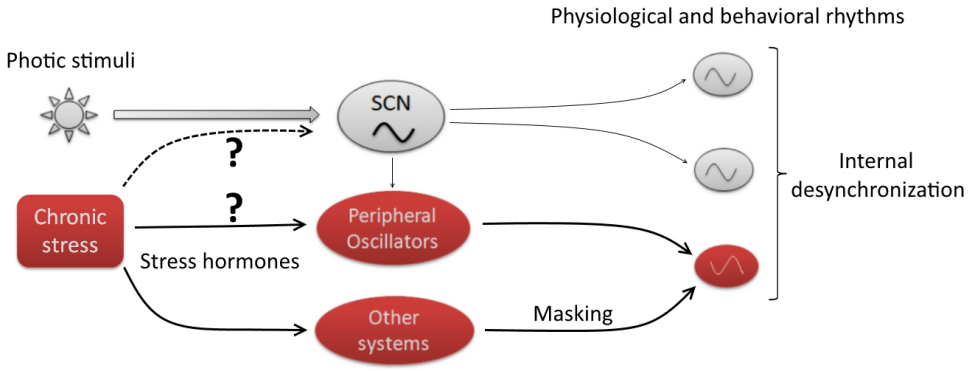

Figure 2. Chronic stress effects on circadian rhythms. The master clock in the SCN is synchronized by the external light-dark cycle, but it does not seem to be affected by stress stimuli. On the other hand, stress hormones are reported to shift phase of clock genes on peripheral tissues which could result in internal desynchronization between different tissues.

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Chapter 2

Effects of stress and stress hormones on endogenous clocks and circadian rhythms: a review

Simone Marie Ota, Deborah Suchecki, Peter Meerlo

This manuscript is part of a review in preparation to be submitted for publication.

Abstract

In mammals, circadian rhythms are under the control of a master clock or oscillator situated in the suprachiasmatic nuclei (SCN). This master clock coordinates peripheral oscillators present in other tissues, maintaining the rhythms synchronized to the main environmental time cue, the light-dark cycle. In contrast, the SCN seems to be well protected against non-cyclic and unpredictable stimuli, such as stress. Studies with social stress in rodents have demonstrated that although it can cause severe disruptions on the shape and amplitude of body temperature and locomotor activity rhythms, the phase and period of these rhythms are not affected, indicating that the SCN is not perturbed. However, the expression of the rhythms is not only determined by the SCN, and the disturbances observed might be due to masking effects of stress on the output rhythm or due to effects on oscillators in peripheral tissues, which are normally under control of the SCN. This disruption between the master clock, peripheral oscillators and physiological and behavioral rhythms may have consequences for health.

1. Introduction

In mammals, daily rhythms can be observed in almost every function and process, ranging from overt behaviors such as locomotor activity, sleep and feeding to physiological measures such as heart rate, body temperature and hormone release. In most cases these daily rhythms are driven by endogenous biological clocks or oscillators that reside in different body tissues (Dibner et al., 2010).

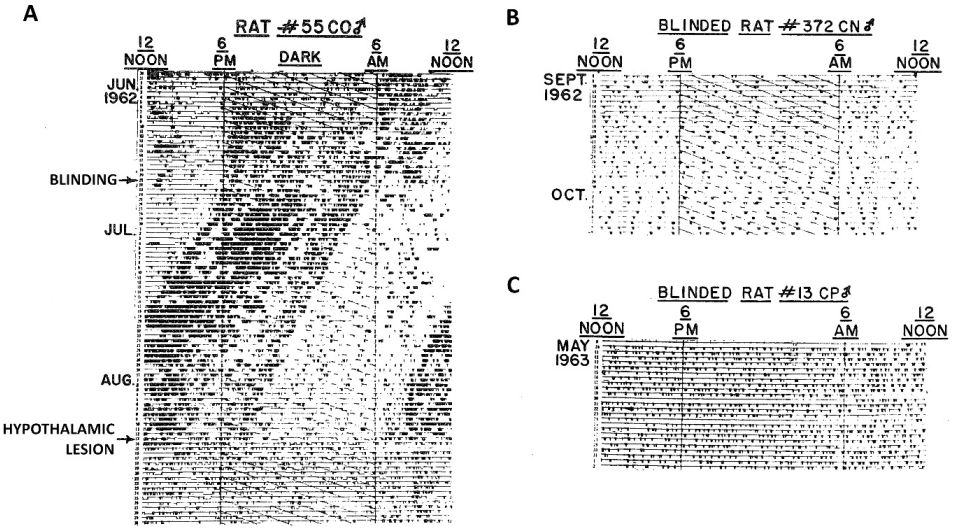

Figure 1. Actograms showing the effect of hypothalamic lesion in blinded rats in A) running rhythm; B) eating rhythm C) drinking rhythm. Modified from Richter, 1967.

In the early 1960's, Curt Richter, at the Johns Hopkins Medical School in Baltimore, demonstrated for the first time the presence of an endogenous clock in the hypothalamus responsible for driving and controlling the daily rhythms in behavior of rats (Richter 1967). He showed that blinding rats and thereby disconnecting them from the environmental light-dark cycle resulted in free running activity rhythms with periods that were most often slightly shorter or longer than 24 h. According to his narratives, the clock system driving these endogenous activity rhythms were largely unaffected by ablation of almost every part of the brain down to the hypothalamus. However, when he lesioned the hypothalamus, rhythmicity in activity would cease to exist all together and all locomotor activity, feeding and drinking became evenly distributed across the 24 h cycle (figure 1). Later, lesion studies narrowed down the location of this clock to a specific sub-region of the hypothalamus, that is, the suprachiasmatic nuclei (SCN) situated right above the optic chiasm (Moore and Eichler 1972, Stephan and Zucker 1972).

The endogenously controlled daily rhythms are generally called circadian rhythms, referring to the fact that the endogenous free-running period of these rhythms is 'about

a day' but often slightly deviating from 24 h (Latin: circa = about, dies = day). As the observations in blinded rats by Richter already suggested, the hypothalamic clock uses light to adjust the endogenous period to exactly 24 h and precisely synchronize the endogenous rhythms to the light-dark cycle in the outside world (Pittendrigh, 1981). To achieve this, photic information is transmitted to the SCN via a direct neuronal input from the retina in the eyes, the retino-hypothalamic tract (Moore and Lenn, 1972). While other environmental factors may influence circadian rhythmicity, there is a general consensus that in mammals the daily light-dark cycle is the most important time cue or 'zeitgeber' for synchronization of endogenous rhythms to the external environment (Pittendrigh, 1981).

The SCN is not the only clock or oscillator in the body. In fact, nowadays it is often thought that every organ and tissue, and perhaps every cell, may have its own endogenous oscillatory activity (Balsalobre 2002, Dibner et al., 2010). For example, spontaneous and near 24 h rhythms have been observed in isolated liver, heart and kidney tissue (Yamazaki et al., 2000; Yoo et al., 2004). The rhythmicity in those tissues are usually assessed by observing the expression of the so-called clock genes, which are found to be expressed at the cellular level, and this molecular clock is autoregulated by negative feedback loops. Briefly, the transcript factors CLOCK and BMAL1 activate the transcription of other clock genes, such as *Per1*, *Per2*, *Cry1* and *Cry2*. In the core feedback loop, PER and CRY proteins form complexes that inhibit the activity of CLOCK and BMAL1. (Mohawk et al, 2012, Bollinger and Schibler, 2014). Hence, daily or circadian rhythms in the mammalian body are the result of a complex constellation of interacting oscillators. In this growingly complex circadian system, the SCN is considered to be the master clock that fine-tunes the various rhythms among each other and also synchronizes them to the environmental day-night cycle (for review, see Mohawk et al, 2012).

It is not difficult to imagine that a disruption of circadian organization and disturbance of precisely tuned rhythmic processes can lead to malfunction and disease. Indeed, this notion is supported by numerous studies on the consequences of shift work and jet lag, conditions that represent a mismatch between the endogenous circadian system and the environment. This may result not only in a disrupted relationship between endogenous rhythms and the external world, but also in disruption relationship between the endogenous rhythms among each other. Such a state of internal desynchronization is likely to have an impact on health and, indeed, chronic shift work has been identified as a risk factor for incidence of colon and breast cancer, metabolic changes, sleep alteration and fatigue (Haus and Smolensky., 2006, Reinberg and Ashkenazi., 2008).

In the same context of a relationship between circadian organization and health, it is an important question whether the endogenous circadian system is sensitive to disturbance by stress. Conditions of uncontrollable and chronic stress are considered to be triggers for disease of which, many are associated with strong alterations in daily rhythms in behavior and physiology (e.g., disturbed sleep-wake rhythm, disturbed rhythms in metabolism and

food intake, disturbed neuroendocrine rhythms). One might thus argue that disruption of circadian organization could be one important underlying mechanism of stress-related disorders such as cardiovascular diseases and psychiatric disorders. In this chapter, we discuss the available literature on the effects of stress and stress hormones on endogenous clocks and circadian rhythms.

2. Stress and stress hormones

In mammals, two main systems take action under stressful situations: the autonomic sympathetic-adrenal-medullary (SAM) system and the hypothalamic–pituitary–adrenal (HPA) axis, which are involved in metabolic and physiologic regulation (Axelrod and Reisine, 1984; Johnson et al., 1992; Ulrich-Lai et al., 2009). Activation of the SAM system stimulates the adrenal glands to secrete adrenaline and noradrenaline, which are involved in the "fight or flight" response. Although peripherally secreted catecholamines are unable to cross the blood-brain barrier, and, therefore, reaching the brain, activation of the locus coeruleus (LC), leading to secretion of noradrenaline in the brain, parallels adrenal activity (Svensson, 1987).

From the other branch of the stress response, the paraventricular nucleus of the hypothalamus (PVN) produces and releases corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). CRH stimulates secretion of adrenocorticotropic hormone (ACTH) by the anterior pituitary gland and this action can be amplified by AVP. ACTH, in turn, is transported in the blood circulation and reaches the adrenal gland inducing glucocorticoid (GC) synthesis and secretion (for review, see Papadimitriou and Priftis, 2009). These hormones participate in metabolic control, cardiovascular activity, and immune response, among other functions, by binding to mineralocorticoid receptors (MR) and glucocorticoid receptors (GR), the latter being more activated in stressful situations and conditions in which GC levels are high, and are also involved in the negative feedback regulation of the HPA axis activity (de Kloet, 2014). Glucocorticoid receptors, which are widely distributed throughout the body, are activated and stimulate or inhibit the transcription of many genes by binding to glucocorticoid responsive elements (GRE) in the promoter region of several genes, including clock genes (Balsalobre et al., 2000).

3. Stress effects on rhythms: changes in clocks or masking?

As will be discussed in the next sections, changes in physiological and behavioral rhythms following some form of stress have been reported in numerous studies. One important issue to always keep in mind is that changes in the shape of a rhythm do not necessarily reflect changes in the circadian oscillatory mechanism involved in regulating these rhythms. It may very well be that the underlying endogenous oscillators or clocks are unaffected but that only their output is masked by alterations elsewhere in the brain or body (Hiddinga et al. 1997; Meerlo et al. 2002, Rietveld et al. 1993). The shape of the

body temperature rhythm, for instance, can be modified by a variety of exogenous and endogenous factors independent of the circadian system including, for example, ambient temperature, meals and food digestion, activity and sleep. Some people may prefer to take a warm shower upon awakening in the morning whereas other people prefer to do this before bedtime in the evening. Such difference in preference can lead to quite different body temperature profiles unrelated to the circadian clock system regulating body temperature. More relevant to the context of this review, experiencing stress often induces an acute increase in body temperature and sometimes even long-lasting changes in the temperature rhythm, but such changes may not indicate an altered circadian regulation of this rhythm (Meerlo et al. 2002). Clearly, the shape and amplitude of most rhythms are not exclusively determined by the circadian system and the rhythms that are measured most often represent a combination of circadian and non-circadian processes. Therefore, to be able to draw conclusions on whether or not differences in the shape of a rhythm are truly related to changes in circadian organization, one really has to study features that are characteristic and specific of the endogenous oscillators. For example, one commonly used approach is to keep organisms under constant conditions or so called 'free-running' conditions when intrinsic circadian features such as the period and phase can be measured (see Figure 3). Nowadays, another procedure to directly probe circadian function is to assess the expression of clock genes in different organs and tissues.

4. Stress-induced changes in rhythms: stress or arousal?

An important consideration that relates to the definition of stress is the fact that arousal is a concept that may partly overlap with stress but is not necessarily the same thing. This is an important issue because studies in laboratory rodents, particularly hamsters, have reported pronounced alterations in circadian function in response to arousing stimuli. These circadian effects of arousal may sometimes erroneously be interpreted as effects of stress, especially when the stimulus or condition inducing the arousal at first glance appears to be aversive. A good example are the studies on the effects of social conflicts in hamsters on circadian rhythmicity. In some of these studies, male hamsters were placed together for 30 min, unless serious aggression occurred, in which case the animals were separated as soon as fighting erupted (Mrosovsky 1988, Refinetti et al. 1992). Actual fighting was thus prevented but the strong tendency for aggression suggests that the interaction may have been perceived as stressful either way. Interestingly, the social interactions resulted in a pronounced shift of the circadian activity rhythm in one study (Mrosovsky 1988) but not in the other (Refinetti et al. 1992). In the first study, hamsters that were returned to their home cage after the interaction in most cases displayed a period of intense wheel running indicating a high level of arousal but the animals in the second study did not consistently ran in their wheel. From other studies in hamsters we know that wheel running is a potent modulator of the circadian organization and can result in pronounced phase shifts (Mrosovsky 1996).

Hence, it seems that it was not the potentially adverse and stressful social conflict itself that resulted in a shift of circadian rhythms, but the arousal associated with wheel running afterwards. Even though wheel running may be associated with physiological activation and release of classical stress hormones, it is not a cognitive stressor in the sense of being an uncontrollable and unpredictable adverse condition (for further discussion, see Koolhaas et al., 2011). Quite the opposite, the hamsters chose to run in the wheel and they might in fact done so because it is rewarding and, if anything, a positive experience (Novak et al., 2012).

Additional evidence that activity affects the clock comes from a report on blind female rats displaying shorter free-running period when given access to running wheel (Yamada et al., 1988) and the same effect being observed in male mice when the activity is concentrated in the beginning of the subjective dark phase, whereas the period is increased when the activity is concentrated at the end of the subjective dark phase (Edgar et al., 1991).

5. Potential stress input pathways to the SCN

One way to address the question of whether the master clock in the SCN might be sensitive to stress is to determine the expression of receptors for classical stress signals, e.g., from the HPA axis and the sympathetic nervous system.

Glucocorticoid receptors (GR) have been detected in infant rats, at postnatal day 2 and 8, but they are less present at postnatal day 12 and 16 and are not observed by postnatal day 20 and adult rats (Rosenfeld et al., 1988). The CRH receptor 1 (CRH-R1) is expressed in the SCN, suggesting reciprocal projections from the PVN (Campbell 2003); however, retrograde tracing markers does not show PVN inputs to the pacemaker (Moga and Moore 1997) nor CRH cells or fibers have been found in the borders of the SCN, at least, in ground squirrels (Reuss et al., 1989). It is, therefore, uncertain whether the SCN is indeed sensitive to CRH input and, if so, where this input would be coming from.

With regards to input from the sympathetic nervous system, Legoratti-Sanchez and colleagues (1989) identified a possible bidirectional communication between SCN and LC by recording evoked potential in the SCN of rats after LC stimulation and vice-versa. When the SCN or LC is electrolytically destroyed, the evoked potential is no longer observed after stimulation of one of the areas. Furthermore, catecholamines modulate the expression of some clock genes (Terazono et al., 2003), and α1 and α2-adrenoceptors are found in the rat SCN, as shown by prazosin and para-aminoclonidine binding in autoradiograms (Morien et al., 1999). However, there is no histological evidence for this SCN-LC direct connection, suggesting the existence of a multisynaptic pathway (Legoratti-Sanchez et al., 1989).

While these studies suggest the existence of a potential input from the sympathetic nervous system to the master clock in the SCN, it is uncertain whether activation of this pathway occurs under conditions of stress and how that affects the activity of the SCN.

6. Stress effects within the SCN

Several studies have reported changes in gene expression within the SCN in response to a variety of stimuli that may be classified as stress.

Rats exposed to acute stress, 30 min restraint or 15 min of forced swim at different times of the day (ZT 3, ZT 5 and ZT 8) do not exhibit PER1 alterations in the SCN (Al-Safadi et al., 2014). Although acute stress does not seem to affect the master oscillator, recent studies show that chronic stress can alter clock gene expression in the SCN. It was observed in pups, whose SCN still express GR until the first postnatal week, that maternal separation during the light phase for 6 days phase shifts the rhythm of Per1 and Per2 expression (Ohta et al., 2003).

In the SCN of adult rats, stress seem to affect the amplitude, but not the phase or period of PER2 rhythm. After 18 days of chronic social defeat in the dark phase the amplitude of PER2 rhythm is increased (Bartlang et al., 2014). However, after 4 weeks of chronic unpredictable stress or 7 days of 3 h of restraint stress at ZT 6, amplitude of PER2 expression is reduced (Jiang et al., 2011; Kinoshita et al, 2012, respectively). After seven days of predator-scent stress, Per1 and Per2 mRNA expression is increased at ZT 19 and decreased at ZT 13 (Koresh et al., 2012). Therefore, it appears that chronic stress can modulate the amplitude of clock gene expression in the SCN in a time-dependent fashion.

Another way to evaluate whether stress signals can reach the SCN is to observe if the production and release of some neurotransmitters are altered. For example, AVP, which is also synthesized by neurons of the dorsomedial part of the SCN, is increased after 10 min of forced swimming or active shock avoidance training (Engelmann et al., 1998 and Biemans et al., 2003, respectively). After one session of scrambled footshock, AVP mRNA is enhanced in the SCN, but vasoactive intestinal peptide (VIP) mRNA, produced by neurons in the ventromedial region of the SCN, is decreased in rats (Handa et al., 2007). Furthermore, in adrenalectomised (ADXed) rats, chronic administration of GC in the light phase enhances AVP mRNA expression and abolishes VIP mRNA rhythm in the SCN (Larsen et al., 1994). However, as injections of AVP do not induce phase shifts (Albers et al., 1984), AVP response may not be a signal of rhythm disturbance.

7. Stress does not affect period and phase of output rhythms controlled by the SCN

The early pioneering studies of Curt Richter in the 1960's are not only of general interest to the field of chronobiology but also of particular interest for this review because his attempts to unravel the mechanisms and conditions controlling and modulating endogenous circadian rhythms included a wide range of manipulations that can be considered as rather severe stressors (Richter 1967). For example, he subjected rats to forced swimming, restraint and electrical shocks, often repeatedly and for several days in a row. Such prolonged exposure to severe stress resulted in a strong suppression of activity and in some cases, rhythmicity was hardly visible. Yet, upon cessation of stress exposure, activity would gradually normalize

and more or less resume at the expected time, indicating that the period and phase of the endogenous clock had not been affected (Richter, 1967). It appeared that the clock had kept ticking at the same pace throughout the days of stress. Only its output had been temporarily masked. One may argue that the methods and analyses Richter used in those days were not highly sophisticated; yet, later studies with different approaches and more detailed analyses did little more than largely confirm his conclusions.

Several studies in rats have shown that social conflicts and defeat by an aggressive conspecific result in severe disruption of daily rhythms in locomotor activity, heart rate and body temperature (Meerlo et al. 1996, Meerlo et al. 1999, Tornatzky and Miczek 1993; also see Figure 2). Although repeated exposure results in more pronounced rhythm changes, even a single social defeat stress leads to disrupted rhythmicity lasting for days up to weeks after the actual social interaction (Meerlo et al. 1996, Meerlo et al. 1999). There is some evidence that the most pronounced rhythm changes occur in animals that do not counterattack in a fight, in line with the view that the stress experience and its subsequent consequences are determined by the perception of uncontrollability (Meerlo et al. 1999). A number of studies specifically addressed the question of whether the changes in activity and body temperature rhythm that result from uncontrollable social stress were a consequence of alterations in the endogenous circadian timing system. In one study, rats were subjected to social defeat stress in the first half of the activity phase (Meerlo et al. 1997), whereas in another, social defeat took place in the middle of the resting phase (Meerlo and Daan 1998). Neither study found an effect of stress on phase or period of the free running rhythms under constant conditions (Meerlo et al. 1997, Meerlo and Daan 1998). In line with Richter's earlier conclusions, these findings suggest that severe social stress does not affect the endogenous circadian clock that drives the rhythms in activity and temperature. Although its output may become masked by stress-induced disturbances elsewhere in the body, the central pacemaker in the SCN appears to be unaffected.

In agreement with this conclusion is a study in which mice were subjected to a more chronic intermittent protocol consisting of a variety of daily stressors including pair housing with unfamiliar males, a social stressor, forced swimming, and restraint. Activity was temporarily suppressed but when the animals were transferred from a light-dark cycle to constant darkness, the free-running period of the activity rhythm in stressed animals was not different from that in unstressed control animals (Solberg et al., 1999). In another study, the effect 30-min daily immobilization stress on free-running activity rhythms in rats under constant conditions was investigated over a 93-day period and compared with the milder 30-min sessions of novelty exposure or brief handling (Barrington et al. 1993). Although 20 to 30% of the rats showed mild changes in the circadian period throughout the experiment, there were no differences among the groups. Since the changes in period only occurred in a small fraction of the animals and were unrelated to a specific stressor, they may very well have been spontaneous drifts in period. Therefore, this study does not provide convincing

evidence for changes in circadian function, even with repeated stress exposure over a period of several months. On the other hand, after chronic social stress, defeated mice lose the first activity bout during the subjective dark phase and display shorter activity and body temperature periods compared to control mice during the 14 days after the stress protocol. These disturbances are observed in wildtype but not in clock gene Per1/2 double mutant mice (Bartlang et al., 2015). Indeed, rhythm disturbances during stress exposure were observed in wildtype and mutant mice. However, the long-term effects observed in animals defeated in the beginning of the dark phase were not seen in the defeated mutant mice and the effects were mild (0.2 h shortened period) and only observed in the C57BL/6N strain. Moreover, since the animals were defeated under light-dark conditions and transferred to constant darkness after the defeats (for half of the animals), the effects could be related to changes of the light conditions and modulation of light sensitivity.

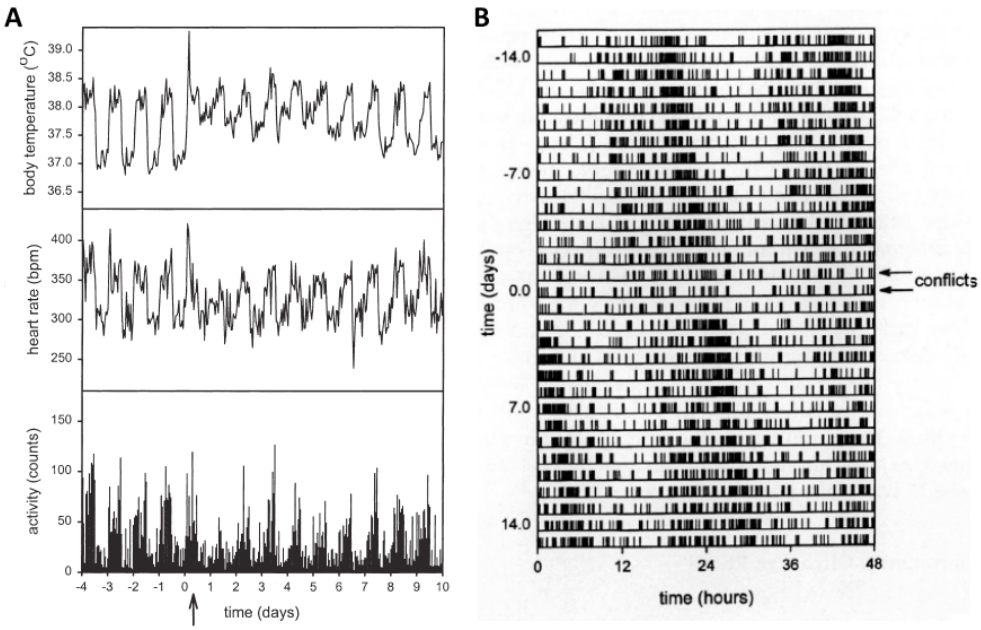

Figure 2. Effects of social defeat in circadian rhythms in rats. (A) Double-plotted actogram of an individual rat showing activity rhythm under constant conditions. No difference was observed in period before and after the two consecutive conflicts, or between control and defeated groups. (B) Body temperature and activity rhythms of an individual rat. Amplitude of both body temperature and activity rhythms decreased after one conflict but returned to baseline levels after about 10 days. Images from Meerlo et al. (1997) (A) and Meerlo et al. (2002) (B).

In addition to the studies that assessed the effects of stressful stimuli or events, some papers have reported on the effects of direct administration of stress hormones or

manipulations that prevent the effects of endogenous stress hormones. Some manipulations, for instance, can affect the output rhythm, but not for long. Intracerebroventricular (ICV) administrations of CRH in the early light phase decreases the amplitude of the activity rhythm in hamsters (Seifritz et al., 1998) and repeated ICV injections over 10 days seems to enhance activity in rats during the night, but does not affect body temperature rhythm (Buwalda et al., 1998). Removal of corticosterone by means of adrenalectomy (ADX) decreases wheel running activity, but does not affect the rhythm, as it remains concentrated in the dark phase. Moreover, administration of dexamethasone (DEXA), a synthetic GC, to ADXed rats, in the beginning of the light or the dark phase, increases running wheel activity after injection, without changing the phase of the circadian rhythmicity (Moberg and Clark, 1976). Neither continuous release nor a 6 h cortisol daily pulses affect the period or phase of the free-running activity rhythm in ADXed hamsters (Albers et al., 1985). Although ADX does not affect synchronization of wheel running activity to a steady 12:12 light-dark (LD) cycle, the lack of corticosterone significantly shortens the time the animals need to resynchronize to an inverted LD cycle (Sage et al., 2004). In addition, corticosterone replacement by subcutaneous pellets with different concentrations does not affect the speed to resynchronization, but rats with an inverted corticosterone rhythm (i.e., ADX rats with access to corticosterone drinking solution only during the light phase) resynchronize more slowly (Sage et al., 2004). Therefore, even though GCs do not directly affect the SCN, they might indirectly influence the way the SCN perceives light information, for example, by modulating tryptophan hydroxylase in the raphe nuclei (Clark et al., 2008), which in turn, modulates the retino-hypothalamic transmission to the SCN (Pickard and Rea, 1997). On the other hand, one study showed that severe social defeat stress did not alter sensitivity to the phase-shifting effect of light (Meerlo et al. 1997).

Altogether, the available literature does not provide strong evidence that acute stressful stimuli or stress hormones perturb the central circadian oscillator in the SCN and more studies are needed on the effects of chronic stress.

8. Stress effects on peripheral oscillators

The data presented thus far suggest that the circadian master clock in the SCN is rather well protected against any disturbance by stressful stimuli. An important remaining question is whether stress or stress hormones can affect other clocks or oscillators that are known to reside in various tissues throughout the body, which are normally under control of the central pacemaker in the SCN (Balsalobre 2002, Dibner et al., 2010).

A single social defeat plus 8 h of sensory contact induces advance of the PER2 peak phase in the adrenal but not in the pituitary gland in mice, whereas chronic subordination for 14 days induces a phase advance in both glands. Therefore, according to the authors, stress may produce effects depending on the duration of stress exposure, first affecting the more sensible adrenal clock, and chronically, altering the pituitary clock (Razzoli et al 2014).

However, in this study, no differences were observed in the locomotor activity rhythm after chronic subordination.

A recent study showed that DEXA altered rhythmic gene expression in cell cultures from liver, kidney, and heart tissue, even though it did not affect gene expression in neurons of the SCN (Balsalobre et al. 2000). Likewise, adrenaline injections in vivo or dissolved adrenaline in vitro increase Per1 expression in the liver (Terazono et al., 2003) and a combination of adrenaline and noradrenaline phase shifts the expression of Per1, and its regulating transcription factors genes E4bp4 (E4 promoter-binding protein) and Dbp (D-box binding protein), in aortic cells in vitro (Reilly et al., 2008).

The reported effects of ADX on circadian rhythms are contradictory in the literature, insofar as ADX delays Per1 phase in the kidney, liver, cornea and pituitary gland, but not in the SCN, lung, pineal or salivary gland, and treatment with hydrocortisone after ADX produces tissue-dependent effects and phase-advance in the SCN (Pezuk et al., 2012). However, Soták and colleagues (2016) did not find phase-shifts after ADX, but only tissue-specific differences in clock gene mRNA levels. The authors hypothesized that some clock genes may be regulated by GCs in a posttranscriptional way. Rhythmic expression of PER2 in the bed nucleus of the stria terminalis and in the lateral division of the central nucleus of the amygdala is abolished in ADXed animals, suggesting that PER2 expression in the limbic system is regulated by rhythmic variation of GCs, since constant release of the hormone, accomplished by pellet replacement does not restore PER2 rhythm in ADX animals (Segall and Amir, 2010).

On the one hand, acute stress appears to have transient effects on clock gene expression, on the other hand chronic stress can reset the phase of peripheral clocks. Restraint stress for 1 h enhances Per1 mRNA in mouse liver, heart, lung and stomach, without any alteration in other clock genes and in locomotor activity (Yamamoto et al., 2005). Restraint stress at ZT 4-6 for 3 days/week, for 4 weeks, induces PER2 rhythm phase advance in peripheral tissues (kidney, liver and submandibular gland) (Tahara et al., 2015 – Figure 3). Social defeat for 3 days also induces PER2 phase-advance in kidney, liver and submandibular gland. These effects seem to be mediated by the HPA and/or SAM axes, since administration of DEXA, noradrenaline or adrenaline also produce phase-advance in PER2 rhythm (Tahara et al., 2015).

Alternatively, temperature may also affect circadian rhythms, as ambient heat pulses are able to phase-shift activity rhythms in rats (Francis and Coleman, 1997). In vitro experiments also confirm that temperature is a resetting signal for peripheral oscillators, as demonstrated by heat pulses of 1 h or 6 h from 36 to 38oC in pituitary and lung tissues (Buhr et al., 2010).

It is plausible to postulate that certain specific types of stress affect peripheral oscillators, perhaps temporarily disturbing their control by the central pacemaker in the SCN, leading to a state of internal desynchronization. Such internal desynchronization might,

in part, be responsible for the disturbances in overt rhythmicity in body temperature seen after, for instance, social defeat stress.

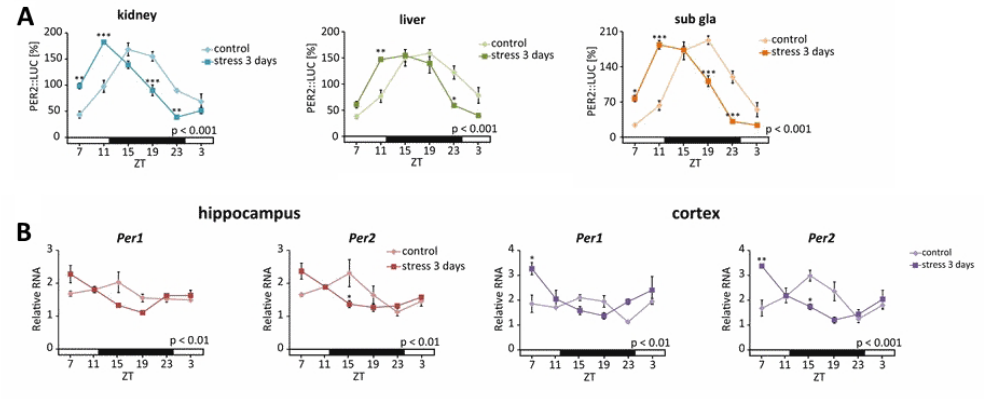

Figure 3. Effects of 3 days of restraint in PER2 expression rhythm in peripheral oscillators. (A) Stress phase advanced expression of PER2::LUC in different peripheral tissues. (B) Stress phase advanced expression of Per1 and Per2 RNA in peripheral oscillators in the brain. Lighter traces represent control and darker traces represent stress groups. Modified from Tahara et al. (2015).

9. Conclusions and discussion

The circadian system has evolved as an adaptation to the highly regular and predictable changes in the environment that are the consequence of the Earths' rotation around its axis. These environmental changes consist of the highly regular alternation of day and night, and often in close association with those daily rhythms in ambient temperature and food availability or accessibility. Endogenously regulated circadian rhythms allow for an optimal temporal organization of behavior and physiology in relation to this cyclic environment. It allows animals to live in synchrony with their cyclic surroundings, and to anticipate and prepare for changes that occur in a predictable daily fashion (Daan 1981; Moore-Ede 1986).

In contrast to the circadian system, the body's stress response systems are an adaptation to the fact that animals are not only exposed to regular and predictable changes in their environment but often have to deal with unexpected threats and challenges (e.g., competitors, predators). In the face of such challenges, a rapid activation of the autonomic sympatho-adrenal axis and the HPA axis, in a complex interplay with various other neuroendocrine systems, allow for acute and adequate response to deal with the unexpected situation at hand (Meerlo et al. 2002). One might say that, whereas the circadian system is an adaptation to predictable aspects of the environment, the stress systems are an adaptation to unpredictable aspects of the environment (Moore-Ede 1986).

From this functional perspective, it makes sense that the circadian timing system

would be thoroughly buffered against effects of unpredictable and uncontrollable stressors that, in many cases, do not contain temporal information relevant for the regulation of daily rhythmicity (Meerlo et al., 2002). In fact, Curt Richter's early studies on rats led him to conclude that the endogenous clock was quite independent form anything that happened in the body (Richter, 1967). More recent work has clearly indicated that his view does not fully hold and that, for example, physical activity or some state of arousal associated with activity provides feedback to the circadian system and is capable of phase shifting the endogenous rhythms (Mrosovsky, 1996). Yet, when it comes to stress, much of the available data from properly controlled and experimental studies in laboratory rodents still suggest that the master clock in the SCN is not disturbed by even severe uncontrollable stressors.

Some contradictory results regarding the effects of stress on circadian rhythms may be explained by the diversity of models studied, by the time of the day when stress is applied and by the length of the stress protocol. Acute stress may suppress locomotor activity and flatten temperature rhythm but does not have a clear effect on phase or period. Therefore, the observed alterations induced by acute stress on these rhythms could be explained by a masking effect on the output rhythms rather than an effect on the SCN. Chronic social stress, in turn, induces a mild effect on period in a specific mouse lineage (Bartlang et al., 2015) and affects the amplitude of clock genes expression in the SCN, but no other major effects are observed. The mechanisms by which stress signals reach the SCN are still under speculation and it may be that other factors, such as activity and temperature, could mediate the alterations.

Administration of stress hormones affects amplitude but does not seem to change the phase or period of output rhythms, only in extreme high doses. Nevertheless, GC and some stress manipulations phase-shift clock genes expression in many peripheral tissues and this effect seems to be tissue specific.

In summary, acute stress does not affect the SCN, but more studies are needed to elucidate the effects of chronic stress. And although the master clock does not seem to be affected by stress and stress hormones, peripheral oscillators may be disturbed, affecting the internal circadian organization and leading to desynchronization-related diseases.

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Chapter 3

Chronic social defeat stress suppresses locomotor activity but does not affect the free-running circadian period of the activity rhythm in mice

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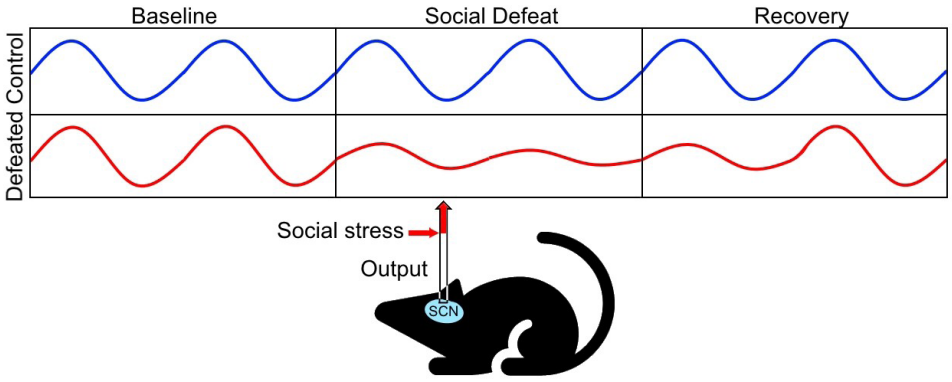
Abstract

In mammals, daily rhythms in behavior and physiology are under control of an endogenous clock or pacemaker located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN assures an optimal temporal organization of internal physiological process and also synchronizes rhythms in physiology and behavior to the cyclic environment. The SCN receives direct light input from the retina, which is capable of resetting the master clock and thereby synchronizes internally driven rhythms to the external light-dark cycle. In keeping with its function as a clock and pacemaker, the SCN appears to be well buffered against influences by other stimuli and conditions that contain no relevant timing information, such as acute stressors. On the other hand, it has been suggested that chronic forms of stress may have gradually accumulating effects that can disturb normal clock function and thereby contribute to stress-related disorders. Therefore, in the present study we investigated whether chronic intermittent social stress affects the endogenous period and phase of the free-running activity rhythm in mice. Adult male mice were maintained in constant dim red light conditions and exposed to a daily 20 min social defeat stress session for 10 consecutive days, either during the first half of their activity phase or the first half of their resting phase. The overall amount of running wheel activity was strongly suppressed during the 10 days of social defeat, to about 50% of the activity in non-defeated control mice. Activity levels gradually normalized during post-defeat recovery days. Despite the strong suppression of activity in defeated animals, the endogenous free-running circadian period of the activity rhythm and the phase of activity onset were not affected. These findings are thus in agreement with earlier studies suggesting that the circadian pacemaker in the SCN that is driving the rhythmicity in activity is well-protected against stress. Even severe social defeat stress for 10 consecutive days, which has a major effect on the levels of activity, does not affect the pace of the endogenous clock.



Graphical abstract

1. Introduction

In mammals, daily rhythms in physiology and behavior are under control of an endogenous clock that is located in the suprachiasmatic nucleus (SCN) of the hypothalamus (Dibner et al., 2010; Saper, 2013). The SCN serves as a pacemaker that directly drives the rhythms or coordinates rhythms that reside in other tissues and organs. The SCN receives a direct neuronal input from the eyes and light is the main time cue used by the SCN to synchronize internal rhythms to the environmental cycles in the external world.

A disturbance in the fine-tuned temporal organization of physiological processes and behavior may have serious consequences for health and well-being. Indeed, desynchrony of internal rhythms has been implicated in a variety of maladies and diseases, including psychiatric disorders, neurological disorders, metabolic syndrome, and inflammation (Jones and Benca, 2015; Maury et al., 2014; Videnovic and Zee, 2015; Wright et al., 2017). Likewise, desynchrony between internal rhythms and the external environment can also cause health problems, as is the case with jet lag and shift work. Shiftwork for example, is associated with sleep-wake problems, fatigue, and poor attention (Caruso, 2015; Herichova, 2013), which can also be observed after long-distance flights across time zones (Samuels, 2012; Weingarten and Collop, 2013).

In this context, it is an important question whether the circadian timing system is sensitive to disturbance by stressors and whether such circadian disturbance might then contribute to the development of stress-related disorders. Many earlier studies have suggested that the SCN, in keeping with its function as a clock and pacemaker, appears to be well buffered against the influence of acute stressors (Meerlo et al., 2002; Richter, 1967). For example, studies in rodents have shown that acute social defeat stress may lead to severe disturbances in the daily rhythms of activity, body temperature and heart rate, but it does not affect the endogenous phase and period of these rhythms under constant conditions (Meerlo et al., 1997b, 1998, 2002). In other words, the endogenous pacemaker driving the rhythms appears to be unaffected, but its output can be masked by disturbances elsewhere in the body. On the other hand, it has been suggested that chronic stress may have more severe effects that accumulate over time and perhaps can disturb normal clock function, if not directly, perhaps indirectly by affecting other systems that communicate with the circadian system (Koch et al., 2017). For this reason, in the present study we assessed the effects of repeated social defeat stress for 10 successive days on free running activity rhythms in mice.

2. Material and methods

2.1 Animals and housing

A total of 45 male C57BL/6J mice (Janvier Labs, Le Genest-Saint-Isle, France) and 15 male CD-1 mice (Charles River, Sulzfeld, Germany) were used for the experiments. The C57BL/6J mice were between 2 and 3 months-old at the beginning of the experiment. They

were used as experimental animals and were assigned to either a control group or a social defeated group. The male CD-1 mice were 3 to 5 months old and were trained to be used as aggressors for the social defeats. All animals were individually housed in cages with running wheels. The mice had free access to food and water throughout the study and the rooms were temperature controlled (21 ± 1 oC). All efforts were made to minimize animal suffering. The experiments were conducted in accordance with the Dutch rules and regulations and approved by the Central Authority for Scientific Procedures on Animals (CCD).

2.2 Experimental Design

Figure 1 shows the timeline of the experiment. After an initial phase of habituation under a standard 12-12h light-dark (LD) cycle, animals were kept under constant dim red light from the start of the baseline period onwards and throughout the remainder of the experiment. Running wheel activity was recorded and compared among the three blocks: baseline, social defeat and recovery; each block consisted of 10 days. In the first experiment, half of the mice were subjected to a social defeat stress during their circadian activity phase for 10 consecutive days. In the second experiment, half of the mice were subjected to a social defeat stress during their resting phase, also, for 10 consecutive days. These time frames for stress exposure were chosen because these are the phases at which the circadian clock in the SCN and the free-running activity rhythm rodents have been shown to be sensitivity to the phase shifting effects of various non-photic stimuli (Mrosovsky, 1996). These phases therefore seemed most relevant in the context of our study on stress. In both experiments, the social defeat stress took place at a fixed external time of day. Because mice were free-running with their own endogenous period that slightly deviated from 24 h, the defeats at fixed external times took place at a slightly different internal time every day.

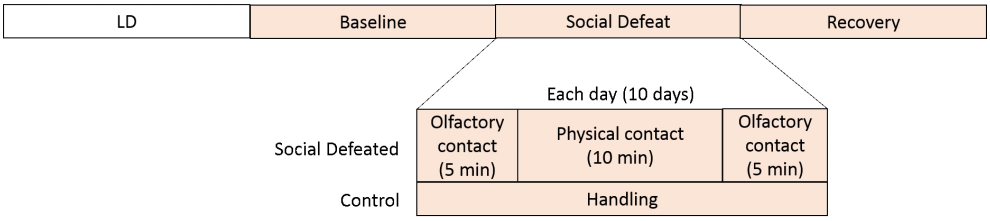


Figure 1. Timeline of the experiment. After entrainment to a 12:12 light-dark cycle, animals were exposed to constant dim red light throughout the rest of the experiment. The timeline of the experiment consisted in three blocks of 10 days each: Baseline, Social defeat and Recovery. During the Social Defeat block, mice from the social defeated group were placed in a cage with an aggressive animal for a total of 20 minutes each day. Control animals were handled and placed in a different cage. During Baseline and Recovery blocks, animals were left undisturbed in their home cages.

2.3 Social Defeat

Social defeat sessions took place under dim red light, similar to that in the home room of the experimental mice, and care was taken to not expose them to any other light. Each social stress session lasted 20 min, and was divided as follows: Phase 1 (5 min) was the initiation phase, during which the experimental animal was placed in the aggressor's cage, separated by a transparent and perforated acrylic partition, allowing olfactory and visual contact. Phase 2 (10 min) was the actual defeat time, which started by removing the partition, after which the aggressor threatened and attacked the experimental animal. If during the interaction phase, the intruder received more than 10 attacks before 10 minutes, the animals were separated and the remaining time was added to Phase 3 (5 min). In phase 3, the mice were separated by the partition again. At the end of the procedure, the intruders returned to their home cage. Social defeated animals were exposed to a new aggressor each day, to avoid habituation. Control mice were placed in an empty cage for the same duration as the defeat procedure.

2.4 Activity Recordings and Data Processing

Running wheel rotations were recorded and stored in 2 min bins throughout the study. The free-running circadian period of the activity rhythm was calculated for each of the 10-day time blocks (baseline, social defeat, recovery) by means of a periodogram analysis based on the Sokolove and Bushell algorithm (ChronoShop 1.04; Spoelstra, 2015). Based on the individual free-running period, the total activity per circadian hour and circadian day was calculated. The phase of activity onset was calculated by a procedure previously described (Meerlo et al., 1997b). Briefly, the time of activity onset was calculated by determining the crossings between a 1h running mean and a 24h running mean of the original raw data. The time of activity onset for the last day of each 10-day block (baseline, defeat, recovery) was then transformed to circadian time, based on the free-running period for each individual mouse.

2.5 Statistics

To assess the effects of social defeat stress on free-running circadian period and phase, repeated measures ANOVA was used with between-subjects factor GROUP (control and social defeat) and within-subjects factor TIME (10-day time blocks for baseline, social defeat, and recovery). To determine differences between the two groups in the overall amount of daily activity, repeated measures ANOVA was applied separately for the three 10-day time blocks with between-subjects factor GROUP (control and social defeat) and within-subjects factor DAYS (10 successive days within a time block). Finally, to assess differences between the two groups in the daily distribution of activity, a repeated measures ANOVA was applied separately for the average daily activity profile in each 10-day time blocks with between-subjects factor GROUP (control and social defeat) and within-subjects factor

HOURS (24 circadian hours). Newman–Keuls test was used as a post-hoc when appropriate. Results were considered statistically significant when $p < 0.05$.

3. Results

3.1 Social defeat during the active phase

Data from two animals in the first experiment had to be excluded because of technical issues with their running wheels and incomplete activity recordings, resulting in a total of 10 and 11 animals in the control and social defeated group, respectively.

Figure 2A shows actograms from an individual control animal and an animal exposed to social defeat stress in the active phase. Figure 2B displays the average circadian period for the control group and defeat group during the three successive 10-day-blocks. There was no difference in free-running period between control and socially defeated animals in any of the 3 time blocks. In the control group, the free-running circadian period for the three successive 10-day blocks was 23.86 ± 0.02 h (baseline), 23.89 ± 0.02 h (experiment), and 23.97 ± 0.03 h (recovery). For the defeated mice, the free-running period was 23.88 ± 0.02 h (baseline), 23.91 ± 0.02 h (social defeat), and 23.92 ± 0.03 h (recovery). Figure 2C shows the average circadian time of the activity onset on the last day of each 10-day block. Repeated measures ANOVA indicated a trend for a GROUP difference ($F(1,19) = 11.87$, $p = 0.06$) and a trend for interaction between GROUP x DAYS ($F(2,38) = 2.93$, $p = 0.07$).

Figure 3A depicts the amount of activity per circadian day and changes herein across the three 10-day time blocks. The amount of daily activity during the 10-day baseline block was not different between the two groups. For daily activity during the 10-day experimental block, ANOVA revealed an overall effect of GROUP ($F(1,19) = 11.87$, $p < 0.01$). Daily activity was strongly suppressed in the socially defeated mice as compared to the control mice. Activity levels in the defeated animals gradually normalized during the first couple of post-defeat days and, overall, ANOVA did not indicate a significant difference between control and defeated mice in the 10-day recovery block ($F(1,19) = 0.94$, $p = 0.34$), although there was a trend for a GROUP x DAYS interaction ($F(9,171) = 1.76$, $p = 0.08$).

Figure 3B shows the average daily activity profiles of the two groups for the three successive 10-day blocks. As expected, the two groups of mice had similar activity profiles during the 10-day baseline block. However, for the 10-day experimental block, repeated measures ANOVA revealed an effect of GROUP ($F(1,19) = 11.88$, $p < 0.01$) and a GROUP X HOURS interaction ($F(23,437) = 3.15$ $p < 0.01$). Post-hoc tests indicated that the socially defeated mice were significantly less active than the controls from CT13 to CT17 (Newman-Keuls, $p < 0.05$ for each time). The average activity profile during the 10-day recovery block did no longer significantly differ between the groups.

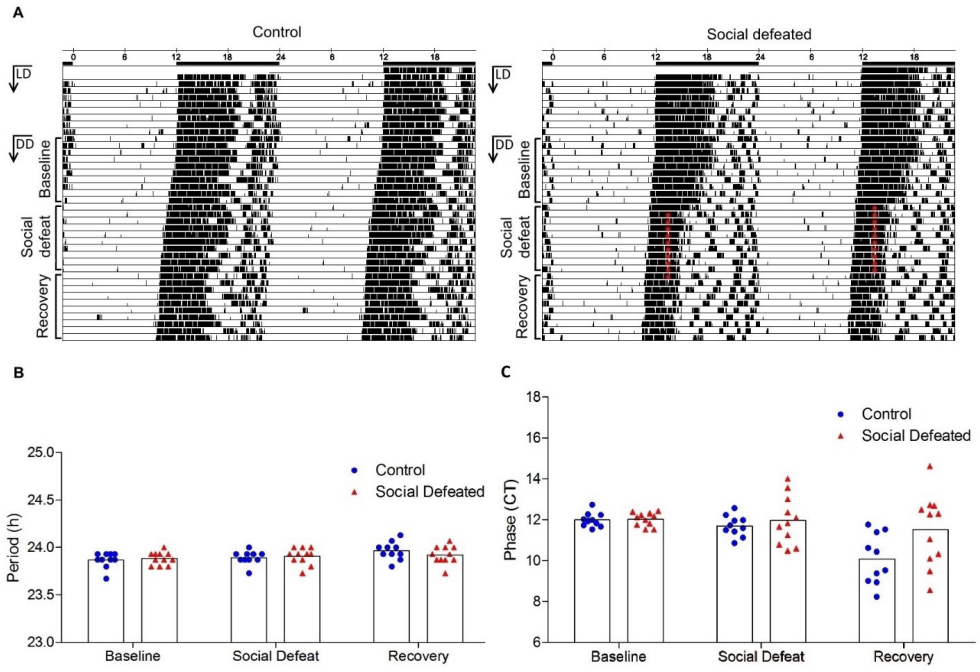


Figure 2. Effects of repeated social defeat stress during the active phase on free-running activity rhythms. Panel (A) Representative actograms of an individual control animal and an animal subjected to social defeat stress on 10 consecutive days during the active phase (indicated by the red line). Observe the suppression of activity that occurred as a consequence of the social defeat stress. Panel (B) The intrinsic circadian period of the activity rhythm during the 3 different phases of the experiment (Baseline, Social Defeat and Recovery). No differences in period between groups were observed. Panel (C) Activity onset phase in the last day of each block of the experiment. No differences between groups were observed. Bars in panel B and C represent means.

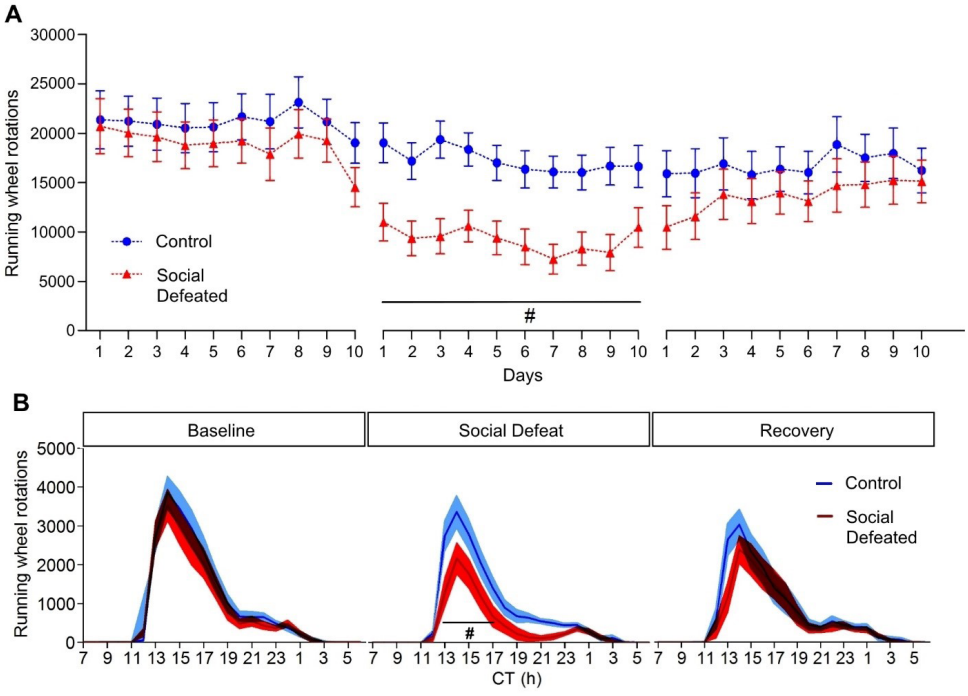


Figure 3. Effects of repeated social defeat stress during the active phase on total activity and distribution of activity. Panel (A) Total activity per day during the three phases of the experiment (Baseline, Social Defeat and Recovery) in control animals (blue lines and symbols) and animals subjected to social defeat stress on 10 consecutive days (red lines and symbols). Animals that were exposed to social defeat showed suppressed running wheel activity compared to control animals. In the Recovery block, there was no longer difference between defeated and control group activity. Data shown are group mean ± SEM. Panel (B) Mean daily activity profile for each 10-day block of the experiment. Defeated mice displayed reduced activity particularly from CT13 to CT17 during Social Defeat days. Lines represent mean and colored area the SEM. For both panel A and B, # indicates a significant difference between groups.

3.2 Social defeat during the resting phase

In the second experiment, data from two animals were excluded due to problems with their running wheels, giving a total of 11 and 9 animals in the control and social defeated group. Figure 4A shows actograms from an individual control animal and an animal exposed to social defeat stress in the resting phase. The free-running periods for the 3 successive 10-day time blocks are shown in Figure 4B. ANOVA did not indicate any difference between control and socially defeated mice for any of these time blocks. In the control group, the free-running circadian period for the three successive 10-day blocks was 23.96 ± 0.03 h (baseline), 23.99 ± 0.05 h (experiment), and 24.01 ± 0.03 h (recovery). For the defeated mice, the free-running period was 23.90 ± 0.03 h (baseline), 23.89 ± 0.06 h (social defeat), and 24.00 ± 0.03 h (recovery). Figure 4C shows the average circadian time of activity onset on the 10th day of each 10-day block. ANOVA did not indicate any difference between control and socially defeated mice.

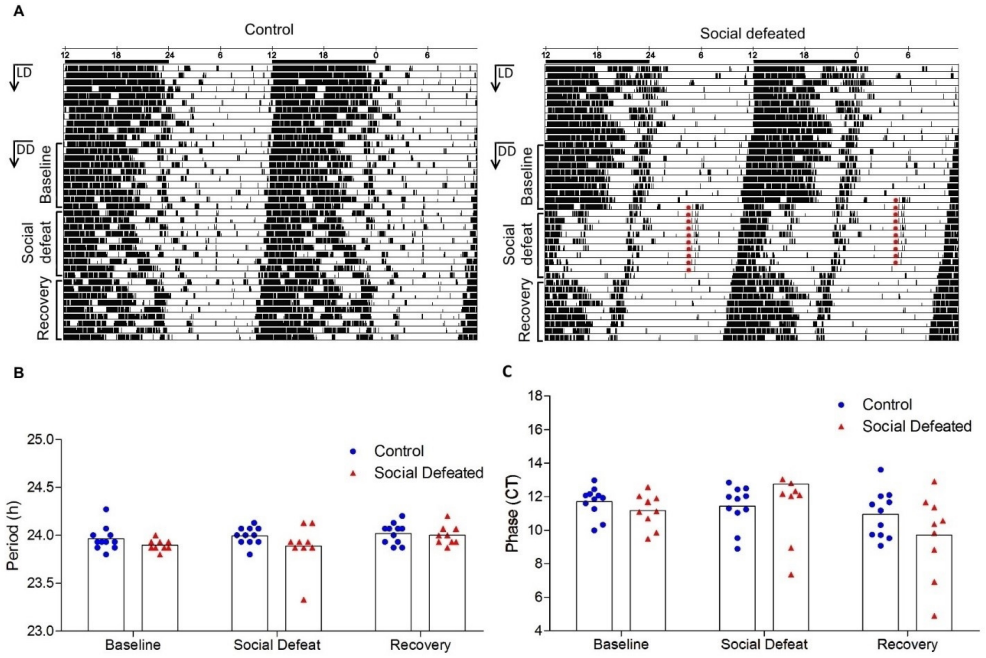


Figure 4. Effects of repeated social defeat stress during the resting phase on free-running activity rhythms. Panel (A) Representative actograms of an individual control animal and an animal subjected to social defeat stress on 10 consecutive days during the resting phase (indicated by the red line). Observe the clear reduction in activity during the active phase of the defeated animal, even though the social conflicts occurred during resting phase. Panel (B) The intrinsic circadian period of the activity rhythms during the 3 different phases of the experiment (Baseline, Social Defeat and Recovery). No differences between the groups were observed. Panel (C) Activity onset phase in the last day of each block of the experiment. There were no differences between groups. Bars in panels B and C represent means.

Figure 5A illustrates the amount of activity per circadian day and changes herein across the three- 10-day time blocks. The amount of daily activity during the baseline was not significantly different between the two groups. For daily activity during the 10-day experimental block, ANOVA indicated that activity in the socially defeated animals was strongly suppressed, relative to the activity level of the control mice (overall effect of GROUP: $F(1,18) = 31.94$ $p < 0.001$). For the 10-day recovery block, ANOVA revealed a significant overall effect of GROUP ($F(1,18) = 7.44$, $p = 0.01$) and a significant GROUP x DAY interaction ($F(9,162) = 4.22$, $p < 0.001$). Activity levels in the defeated animals gradually returned to those seen in control mice but were still significantly lower on the first 2 days of the recovery phase (Newman-Keuls, $p < 0.05$).

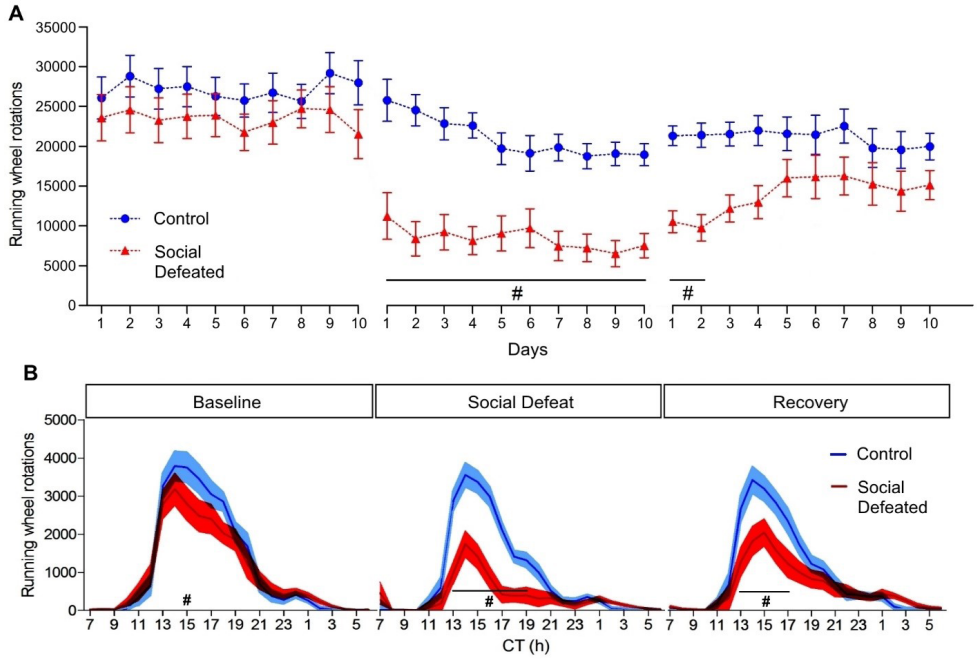


Figure 5. Effects of repeated social defeat stress during the resting phase on total activity and distribution of activity. Panel (A) Total activity per day during the three phases of the experiment (Baseline, Social Defeat and Recovery) in control animals (blue lines and symbols) and animals subjected to social defeat stress on 10 consecutive days (red lines and symbols). Animals that were exposed to social defeat showed suppressed running wheel activity compared to control animals. This suppression gradually disappeared in the course of the recovery phase but was still significantly different during the first 2 days of recovery. Data shown are group mean ± SEM. Panel (B) Mean daily activity profile for each 10-day block of the experiment. During Baseline, the would-be defeated group showed less running wheel activity at CT 15. During the 10-day Social Defeat phase, defeated animals displayed strongly suppressed activity particularly from CT 13 to CT 19. And during the Recovery phase, defeated animals on average still ran less than controls from CT 13 to CT 17. Lines represent mean and colored area the SEM. For both panel A and B, # indicates a significant difference between groups.

Figure 5B shows the average daily activity profiles of the control and defeated mice for the three successive 10-day blocks. The average activity profiles during the baseline period were slightly but significantly different between the groups, as ANOVA indicated a significant GROUP x HOURS interaction ($F(23,414) = 1.67$, $p < 0.05$). Post-hoc analysis indicated that the would-be defeated group ran less than the control mice at CT15 (Newman-Keuls: $p < 0.05$). For the 10-day experimental block, repeated measures ANOVA revealed an overall effect of GROUP ($F(1,18) = 30.61$, $p<0.001$) and a significant GROUP x HOURS interaction ($F(23,414) = 11.50$, $p<0.001$). Post-hoc tests indicated that the socially defeated mice were significantly less active than the controls for a large part of the active phase from CT13 to CT19 (Newman-Keuls: $p < 0.05$ in each case). Even during the 10-day recovery period, the average activity profile of the defeated animals was still significantly different from that of the control mice: ANOVA showed an overall effect of GROUP ($F(1,18) = 7.57$, $p = 0.01$) and a GROUP x HOURS interaction ($F(23,414) = 4.38$, $p < 0.001$). Post-hoc tests demonstrated that defeated animals ran less than controls from CT13 to CT17 (Newman-Keuls, $p < 0.05$ for each time point).

4. Discussion

The results of the present experiments in mice show that exposure to uncontrollable social defeat stress for 10 successive days causes a major suppression of activity levels but does not affect the clock responsible for the rhythmicity in activity: neither repeated defeat in the circadian active phase nor repeated defeat in the resting phase had a significant effect on the endogenous free-running period of the activity rhythm or the circadian time of activity onset.

The suppression of locomotor activity in socially defeated animals is in line with previous studies in rats and mice exposed to acute or chronic stressors (Meerlo et al., 2002; Richter 1967). Particularly, social defeat stress has been found to lead to pronounced reductions in activity that in some cases persist for several days after the last defeat experience (Bartlang et al., 2015; Meerlo et al., 1996, 1997b, 1999). In the present study, the suppression of activity seemed to be slightly stronger and more persistent in mice exposed to defeat in the resting phase as compared to mice exposed to defeat in the active phase. However, since the two experiments were independently carried out in different cohorts of mice, this extrapolation of the findings needs to be considered with care. The social stress-induced suppression of activity may be partly due to a motivation deficit and viewed as a depressive-like behavior, as already observed in social defeated rats (Meerlo et al., 1996b; Rygula et al., 2005) and mice (Krishnan et al., 2007; Kudryavtseva et al., 1991). Another possibility is that the activity reduction could be caused by pain in the defeated mice, since they were bitten by the aggressors. However, besides the caution we took so that the animals would not be injured, another study with social stress showed that avoidance behavior observed in defeated mice was not caused by difference in locomotor

activity, since it was not different from control animals (Krishnan et al., 2007, supplemental data). Another hypothesis is that defeated mice present a higher sleep debt and might be asleep instead of running. To our knowledge, no study has investigated the effects of chronic social stress on the sleep-wake cycle, but studies with one or two defeats showed that sleep debt seemed higher in defeated animals, as they present more slow-wave activity during non-REM sleep (Meerlo et al., 1997; Meerlo and Turek, 2001).

In previous studies, social defeat stress was found to not only affect the activity rhythm but also rhythms in physiology, including body temperature. Social defeat stress is often associated with elevated body temperature during the resting phase that can last for several days after the end of the stressor (Meerlo et al., 1996, 1997b, 1999; Tornatzky and Miczek, 1993). In one study in rats, the reduction in activity after social defeat stress strongly correlated with the increase in resting temperature: the more activity was suppressed during the active phase, the more body temperature was elevated during the resting phase (Meerlo et al., 1996). This finding indicates that the change in activity per se is unlikely to be responsible for the change in body temperature, but the correlated change suggests that the two may share a common mechanism.

One often proposed mechanism for stress-induced changes in activity patterns and physiological rhythms is a disturbance of the circadian system. However, the current study, performed under constant conditions, showed that the circadian pacemaker driving the rhythmicity in activity continued to run at the same pace: despite the suppression of overall activity levels, the free-running circadian period of the activity rhythm and the timing of activity onset was not affected.

The finding that severe stress does not affect the free-running period of the activity rhythm is in agreement with previously published work in rodents exposed to social defeat or other stressors (Meerlo et al. 2002, Richter 1967). A number of studies specifically addressed the question of whether the changes in activity and body temperature rhythm that result from uncontrollable social stress are a consequence of changes in the endogenous circadian timing system. In one study, rats were subjected to social defeat stress in the first half of the activity phase (Meerlo et al., 1997b), and in another study social defeat occurred in the middle of the resting phase (Meerlo and Daan, 1998). In neither one of these studies social stress had an effect on the phase or the period of the free running rhythms under constant conditions (Meerlo et al. 1997b; Meerlo and Daan, 1998). The present study in mice showed that even repeated defeat stress on 10 consecutive days did not have the proposed long-lasting effects that would culminate in an altered period. And overall, there was no significant difference between defeated and control groups in the phase of activity onset, although there was a trend for a difference when animals were defeated in the active phase. In this case however, it seemed that control mice, rather than defeated animals, had a slightly earlier time of activity onset during the last 10-day time block of the experiment compared to baseline.

Bartlang and colleagues recently reported on the effects of chronic intermittent social stress on activity rhythmicity in mice. The animals were exposed to a social conflict for 19 consecutive days, either in the light or in the dark phase, after which they were kept in constant darkness to study their free-running rhythms. In contrast to our findings, they reported a phase delay in peaks of activity in both C57BL/BN mice and C57BL/6J mice, especially in animals defeated in the dark phase. They also found a small but significant shortening of the free-running period of about 10 min in C57BL/BN mice but not in C57BL/6J mice (Bartlang et al., 2015). The change in phase, as the authors discussed, could be explained by a change in the shape of the rhythm, caused by a conditioned fear suppression of the activity, which was also observed in our mice, even when the social defeats occurred during the resting phase. It is unclear why the stress effect on period only occurred in one strain, but it might partly explain the difference from our current study, which was done in the C57BL/6J strain. Interestingly, another study showed that expression of the Period2 clock gene in the SCN was not affected by their protocol of chronic social stress (Bartlang et al., 2014). The latter might suggest that the small change in the period of the activity rhythm observed in one mouse line may be unrelated to the central pacemaker, which is in line with the general picture that the circadian pacemaker is resistant to stress.

A number of earlier studies performed in the golden hamster showed phase shifts in response to a wide variety of stimuli, some of which might be considered stressors, including aggressive social interactions (Mrosovsky, 1988, Mistlberger et al., 2003) and immobilization or restraint (Van Reeth et al. 1991). However, subsequent studies revealed that these phases shifts were not a direct consequence of the stressor but were the result of high intensity wheel running that occurred afterwards (for review, see Meerlo et al. 2002). For example, aggressive interactions between male hamsters induced phase shifts in some studies (Mrosovsky, 1988; Mistlberger et al., 2003), but not in all (Refinetti et al., 1992). In the first studies mentioned, the fighting was consistently followed by a period of running wheel activity, whereas in the latter experiment it was not. It thus seems that the aggressive and presumably stressful interaction only resulted in phase shifts when it induced an increase in locomotor activity. Stress per se did not appear to be the critical aspect of the stimulus. Similarly, immobilization or restraint was sometimes found to be associated with phase shifts (Van Reeth et al. 1991) but also in this case, rather than being the result from restraint stress per se, shifts only occurred when animals displayed wheel running after being released from restraint (Mistlberger et al., 2003; Mistlberger and Antle, 2006). Taken together, these studies in the golden hamster, provide indirect evidence supporting our conclusion that stress by itself does not perturb the central circadian oscillator."

One potential limitation of our study is that we only assessed the effects of repeated social stress at roughly two circadian phases, i.e., the middle of the resting phase and the first half of the active phase. One might argue that stress perhaps could affect the circadian clock and the free-running activity rhythm at other phases. While this needs to be tested, we

specifically chose these circadian phases for stress exposure because these are the phases at which the circadian clock in the SCN and the free-running activity rhythm appears to be most sensitivity to the phase shifting effects of non-photic stimuli discussed in the previous paragraph (Mrosovsky, 1996). These phases thus seemed most relevant in the context of our study on stress.

While the central circadian pacemaker in the SCN may be well-protected against the effects of stress, the possibility that stress can perturb peripheral oscillators that reside in other tissues and organs throughout the body, which are normally under regulatory control of the SCN cannot be excluded. In fact, although chronic social stress in mice did not change Period2 expression in the SCN, it produced a phase advance in the expression of this clock gene in the adrenal glands (Bartlang et al., 2014). Also, treatment with dexamethasone, a synthetic analogue of the glucocorticoid stress hormone, does not affect the SCN, but does shift the rhythms in clock gene expression in liver, kidney, and heart tissue (Balsalobre et al., 2000). These findings are consistent with the fact that glucocorticoid receptors, which are abundantly present in most tissues, are no longer expressed in the adult SCN (Rosenfeld et al., 1988). Hence, through glucocorticoids mechanism, stress might affect oscillatory processes in many tissues while leaving the central oscillator in the SCN untouched. It thus remains possible that changes in the phase relations among multiple clocks in the brain and body underlie some aspects of stress pathology. Therefore, more studies are necessary to assess the effects of different stressors on peripheral oscillators and the mechanisms involved.

In conclusion, while effects of stress on peripheral oscillators need to be investigated, the current study supports our earlier studies showing that acute social defeat stress does not affect the central pacemaker in the SCN. The current experiments extend our earlier findings by showing that even chronic intermittent social defeat stress for 10 days does not affect the free-running period of the activity rhythm that is driven by the master clock.

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Chapter 4

Chronic social defeat stress affects the circadian rhythm of PERIOD2 in the liver but not in the suprachiasmatic nucleus

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This chapter is part of a manuscript in preparation to be submitted for publication

Abstract

Circadian (~24 h) rhythms in behavior and physiological functions are under control of an endogenous circadian clock in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN acts as a pacemaker that drives some of these rhythms directly or serves as a coordinator of peripheral clocks and rhythms residing in other tissues and organs. It has been hypothesized that disruption in circadian organization may contribute to the development of disease, including stress-related disorders. Previous studies on stress, including our own studies on severe social defeat stress in rodents indicate that the mammalian 'master clock' in the suprachiasmatic nucleus of the hypothalamus (SCN) is highly resistant to any effect of stress. However, it is unclear whether stress affects clocks and rhythms in other tissues, which might then lead to a state of internal desynchronization. In the present study, we examined the effect of uncontrollable social defeat stress on the master clock in the SCN and the peripheral clock in the liver. We used transgenic PERIOD2::LUCIFERASE knock-in mice to assess the rhythm of the clock protein PERIOD2 (PER2) in SCN slices and liver tissue collected after 10 consecutive days of social defeat stress. The rhythm of PER2 protein in the SCN was not affected by prior exposure to stress, whereas in the liver PER2 rhythm had a significantly delayed phase in defeated than in non-defeated control mice. This study confirms earlier findings showing that the SCN is resistant to stress, but it also shows that clocks in other tissues can be affected by stress. Future studies are needed to determine the mechanism by which stress affects the liver clock.

1. Introduction

Circadian (~24 h) rhythms are an intrinsic part of mammalian biology, and a wide range of behavioral and physiological functions show significant variation across the circadian cycle (Honma, 2018). Although a fundamentally cellular process based on negative transcriptional feedback loops, (Welsh et al., 2010, Shearman et al., 2000), many circadian functions in mammals are driven through the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN is thought to act as a central pacemaker for circadian timekeeping in mammals, coordinating information about external lighting events (such as the day-night cycle) via the optic nerve, and relaying this to cells and organs in the rest of the body (Rosenwasser and Turek, 2015). For this reason, the SCN is often referred to as the 'master clock'.

Isolated tissue sections, such as, liver, kidney and lung, pituitary, and cornea when maintained in culture, also exhibit circadian rhythmicity of clock proteins, including PERIOD2 (PER2), one of the proteins produced in the core feedback loop of the molecular clock in the cells, even when collected from SCN-lesioned mice. Although relative phase can differ between tissues, they still maintain a consistent period, suggesting that while the SCN is important in synchronizing peripheral tissues with external lighting cues, and for driving rhythms in animal behavior, it is not necessary to generate a coherent circadian rhythm in peripheral tissues (Yoo et al., 2004).

Together the SCN and peripheral cellular oscillators provide a precise circadian organization that is important for optimal performance and health. It has been hypothesized that a disruption of the circadian organization as occurs, for example, during shift work, can have a negative impact on health and eventually contribute to the development of diseases (Hsieh et al., 2014). Along the same lines, it has been argued that circadian disruption as a consequence of stress may be an important mediating factor in the pathogenesis of stress-related disorders (Healy, 1987, Schnell et al., 2014). This latter is partly based on studies showing that stress-related disorders are often associated with changes in some aspects of rhythmicity, such as disturbance of the sleep-wake rhythm, altered temperature profile and changes in the daily pattern of hormone release (Meerlo et al. 2002). However, whether such changes in overt rhythms are truly caused by a disturbance of the endogenous circadian timing system remains uncertain.

In fact, much of the available data suggest that the master clock in the SCN is highly resistant to the effects of stress and stress hormones (Meerlo et al. 2002, Richter 1967). For example, controlled studies in laboratory rats have shown that acute social defeat stress may lead to severe disturbances in the amplitude of daily rhythms of activity, body temperature and heart rate, without affecting the endogenous phase and period of these rhythms under constant conditions (Meerlo et al., 1997, 1998, 2002). Moreover, our recent studies in mice show that even repeated defeat for 10 consecutive days, although leading to a strong suppression of the overall activity, failed to affect the endogenous period and phase of the activity rhythm (Ota et al. 2018, see chapter 3). However, while the SCN may not be

sensitive to disruption by stress, it is still unclear whether stress affects circadian oscillations in other tissues, which might then lead to a state of internal desynchronization with potential detrimental effects for health. There is some existing evidence suggesting that peripheral clocks could be responsive to stress. For example, it has been amply demonstrated that administration of either synthetic and endogenous glucocorticoids can reset the rhythm in clock gene transcription rhythm in peripheral clocks, such as the liver, kidney and heart (Balsalobre et al. 2000).

Since glucocorticoids (GC) are the final product of the stress-responsive hypothalamic-pituitary-adrenal (HPA) axis, it is possible that altered GC rhythmic release, could affect circadian rhythms in multiple tissues and thereby lead to the development of several stress-related diseases. However, previous studies observed that, although stress might suppress activity and change the amplitude of the temperature rhythm, it did not seem to affect the period or the phase of gene expressions rhythms in the SCN, thus this central oscillator may not be significantly affected by stress (Meerlo et al., 1997; Meerlo and Daan, 1998; Ota et al., 2018), possibly because the adult SCN does not seem to express glucocorticoid receptors (GR) (Rosenfeld et al., 1988). Nonetheless, stress could still affect peripheral oscillators and cause internal desynchronization.

Therefore, our aim was to assess the effects of chronic social stress on the circadian activity rhythm and to evaluate whether stress affects the circadian rhythm at the molecular level in the SCN and peripheral clock in the liver. For this purpose, we used transgenic PERIOD2::LUCIFERASE (PER2::LUC) mice, which produce a PER2::LUC fusion protein that allows for prolonged and continuous tracking of PER2 expression by means of measurement of luciferase-driven bioluminescence (Yoo et al. 2004).

2. Material and methods

2.1 Subjects

Twenty male PER2::LUC knock-in mice with a C57BL/6 background (Yoo et al. 2004) from our own colony were used as experimental animals and assigned to either a control group or a social defeat group. The animals were individually housed in cages with a running wheel. Ten male CD-1 mice (from Charles River, Sulzfeld, Germany) were used as aggressors for the social defeats. The CD-1 mice were individually housed in a different room, where social defeats occurred. All mice had free access to food and water throughout the study and the rooms were temperature controlled (21 ± 1oC). The experiments were conducted in accordance with the Dutch rules and regulations and approved by the Central Authority for Scientific Procedures on Animals (CCD).

2.2 Experimental Design

The experimental mice were maintained under a 12:12 LD cycle until the start of the study, when they were transferred to constant dim red light. Running wheel activity was recorded and analyzed for two time-blocks, baseline and stress, each consisting of 10 days.

During the 10-day stress phase, half of the mice were subjected to a daily social defeat. The other half served as control and were picked up and moved to a new cage for the same duration of time (see Figure 1). The daily social defeat stress and control procedures took place at a fixed external time of day. On the day of the first defeat session, this was near the end of the active phase (around CT 23). Because the mice were free-running with their own endogenous period that was slightly longer than 24 h, the defeats at a fixed external time occurred at a slightly earlier internal time every day. One hour after the last defeat, mice were euthanized and SCN and liver tissues were collected for in vitro measurement of PER2 expression.

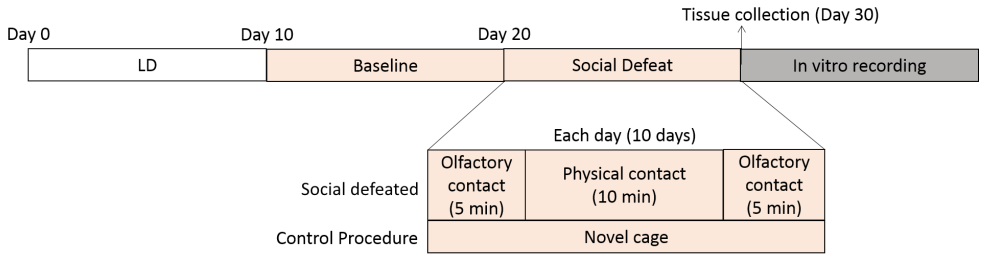

Figure 1. Timeline of the experiment 1. The animals were habituated to a 12: 12 LD cycle for at least 10 days, after which they were transferred to a room with constant dim red light. For the next 10 days, the mice were undisturbed and baseline running wheel activity was recorded. Subsequently, the mice in the Social defeated group were submitted to the social stress once a day, for 10 days, as shown in the scheme, while the control animals were handled and placed in a different cage. After 10 days of social stress, the animals were euthanized and liver and SCN tissues were collected for the in vitro recording.

2.3 Social Defeats

Social defeat sessions took place under dim red light, similar to that in the home room of the experimental mice and care was taken to not expose them to any other light. Each social defeat session had a total duration of 20 min, divided in 3 phases (see Figure 1). Phase 1 (5 min) was the initiation, during which the experimental animal was placed in the aggressor's cage, separated by a transparent and perforated acrylic wall, allowing olfactory and visual contact. Phase 2 (10 min) was the actual phase of physical interaction and defeat that started by removing the wall, after which the aggressor threatened and attacked the experimental animal. If during this phase, the intruder received more than 10 attacks before the end of the 10 minute interval, the animals were separated and the remaining time was added to Phase 3. In Phase 3 (5 min), the mice were separated by the wall again. At the end of the procedure, experimental animals returned to their home cage. Social defeated animals were exposed to a new aggressor each day, to avoid habituation. Control mice were placed in an empty cage during the time the animals from the defeat group were exposed to social stress.

2.4 Tissue preparation and in vitro recording

The procedures for tissue preparation and in vitro measurement of PER2 expression were similar to a previously described procedure, with minor adaptations (Yamazaki and Takahashi, 2005). Briefly, animals were euthanized by decapitation 1 h after the last defeat, still under red light. The eyes were also removed to fully exclude light reception by the retina. The remainder of the procedure was done in the light. Coronal brain sections (200 µm) were cut with a vibratome (CI.7000SMZ, Campden Instruments Ltd., Leicester, U.K.) in chilled Hanks' buffered salt solution (HBSS). Both SCN were later separated from the rest of the brain using a scalpel and placed in a dish with membrane culture inserts and pre-warmed recording medium. A piece of the left lateral lobe of the liver was dissected and slices of approximately 1 mm were cut with a scalpel, also in chilled HBSS. Two liver samples from each animal were taken and placed in separate dishes with pre-warmed recording medium. The medium used in the present study was the same as published standards (Yamazaki and Takahashi, 2005), except that the B27 supplement was substituted by modified NS21 (Crosby et al., 2017) without CORT. The dishes with the samples were placed in the luminometer LumiCycle 32 (LumiCycle, Actimetrics Inc., Evanston, IL) and light emission, as reporter for PER2 expression, was measured for 5 days at 10 minute intervals, at 37°C.

2.5 Data Analysis

Running wheel activity was recorded in 2 min bins and analyzed with ChronoShop 1.04 (Spoelstra, 2015) for calculation of the period using the periodogram analysis based on the Sokolove and Bushell algorithm (Sokolove and Bushell, 1978). The daily onset phase of the activity rhythm was determined using a method similar to that described by Meerlo and colleagues (1997). Activity data was smoothened by a 1 h running average and the activity onset phase was defined as the time the 1 h smoothened data exceeded a 24 h running average. Afterwards, the times were transformed in circadian time (CT) for each animal, based on its period. Total activity per day as well as activity profile were also calculated in excel by aligning the activity counts according to the free-running period for each animal in each 10-day block. We tested the effects of stress on phase and period using repeated measures ANOVA, with between-subjects factor GROUP (Control and Social defeated) and within-subjects factor TIME (Baseline, Social defeat). Repeated measures ANOVA with between-subjects factor GROUP and within-subjects factor DAYS (1-10 day in each block) was used to test the effect on total activity per day. Analysis of the effects of stress on activity profile was done by repeated measures ANOVA with between-subjects factor GROUP and HOURS (24 circadian hours). Newman–Keuls test was used as a post-hoc when necessary. Results were considered statistically significant when $p < 0.05$.

Data analysis included hour 36 to 120 (hour 0 corresponded to start of bioluminescence recording). The first 24 h were excluded because the cellular bioluminescence during this time may exhibit changes related to dissection and media change. The analysis started at

hour 36 due to the method used to remove drift in the average bioluminescence level. The drift in the bioluminescence recording trace was removed (detrended) using a 24 h moving average. The detrended data were then analyzed in GraphPad Prism (version 7.00 for Windows, GraphPad Software, La Jolla California USA) by fitting a cosine wave, accounting for damping of oscillatory amplitude, described by Crosby and colleagues (2017). The period of the fitted cosine was used as a measure for the period of the PER2 rhythm. The phase was determined by selecting the second peak of PER2::LUC rhythm in each sample. When both liver samples for a given animal survived, the results of these two samples were averaged. A Student's t-test was used to analyze the effects of social stress on period and phase of the PER2::LUC rhythm.

3. Results

3.1 Circadian activity rhythm

Data from 5 animals were excluded from the analysis due to technical issues and loss of activity recordings, resulting in a total of 7 animals in the control group and 8 in the social defeated group. Also because of incomplete activity recordings, data from the Social Defeat block was analyzed until the 8th day.

Figure 2A shows examples of activity recordings in a control and a social defeated mouse under constant dim red light; both groups displayed mean free-running periods slightly longer than 24 h (Control = 24.01 h and Social defeat = 24.07 h). Overall, repeated measures ANOVA did not reveal any effect of defeat on free-running period (GROUP $F(1,13) = 3.00$, $p = 0.11$; GROUP x TIME block interaction: $F(1,13) = 2.00$, $p = 0.20$; Figure 2B).

Figure 2C depicts the mean circadian time of activity onset on the 10th baseline day and the 8th day of the Social Defeat block. Repeated measures ANOVA showed no difference between groups ($F(1,13) = 1.12$, $p = 0.31$), neither a GROUP x DAY interaction ($F(1,13) = 0.73$, $p = 0.41$).

The average number of running wheel rotations per day are represented in Figure 3A. There was no difference in daily activity between groups during Baseline ($F(1,13) = 1.06$ $p = 0.32$). During the Social Defeat block, defeated mice ran less than control animals ($F(1,13) = 7.95$, $p = 0.01$). Repeated measures ANOVA also indicated an effect of DAYS ($F(7,91) = 2.19$, $p = 0.04$); however, the post-hoc test did not detect differences among the days during Social Defeat.

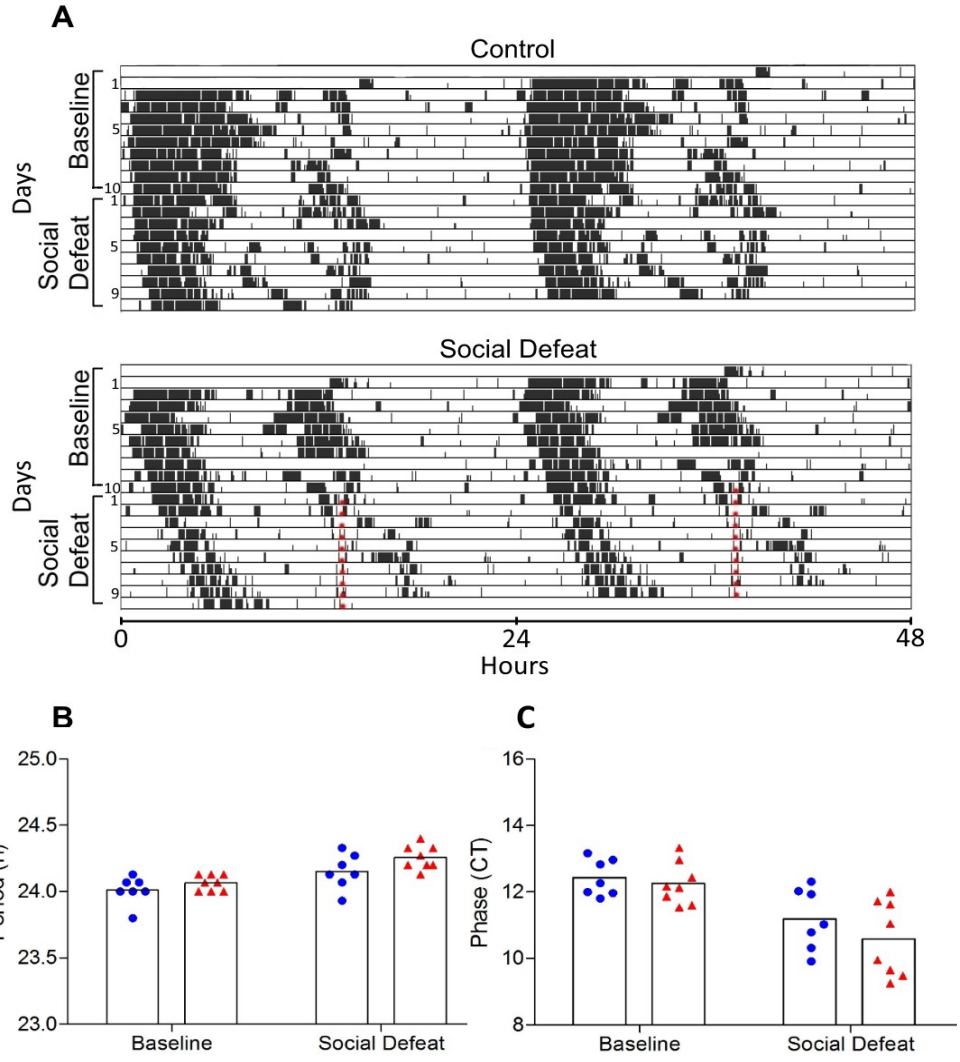

Figure 2. Panel A) Representative actograms of a control and a social defeated mouse. Red dots indicate when handling or social stress occurred. Observe activity suppression, especially in the end of active phase in the second actogram. Panel B) Period of activity rhythm during Baseline and Social Defeat days. Bars represent mean, blue dots represent control animals and red dots represent social defeated animals. Panel C) Activity onset phase during Baseline and Social Defeat days. No significant difference between groups or time was observed. Bars represent mean, blue dots represent control animals and red dots represent social defeated animals.

Figure 3B shows the average 24 h activity profiles of mice during the 10-day baseline block and during the experimental block. There was no difference between the groups during Baseline days. During the social defeat days there was a main effect of GROUP ($F(1,13) = 7.95$, $p = 0.01$) and a GROUP x HOURS interaction ($F(23,299) = 2.63$, $p < 0.01$). The post-hoc test indicated that Social defeated group ran less than Control group from CT 13 to CT 16.

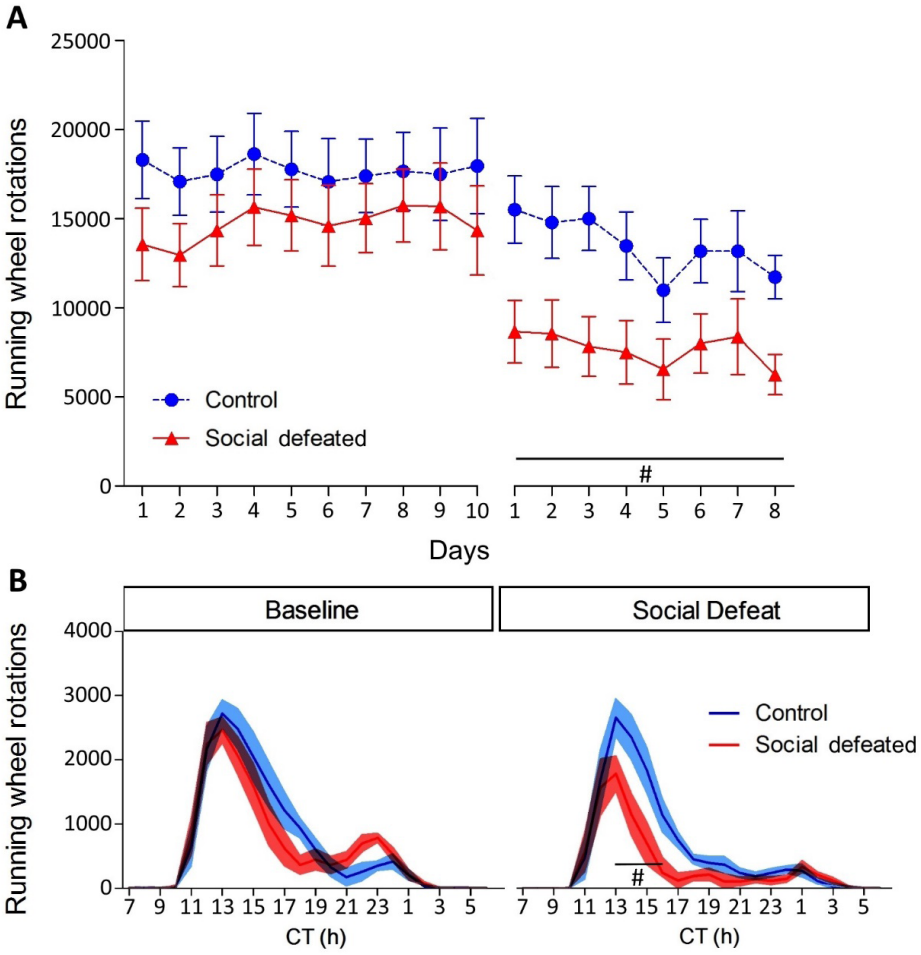

Figure 3. Panel A) Total running wheel rotations per day during Baseline and Social Defeat days. There was a difference between groups during Social Defeat. Symbols represent mean ± SEM. Panel B) Total running wheel activity per hour during Baseline and Social Defeat blocks. The Social defeated group ran less than the Control group between CT 13 and CT 16 during the Social Defeat block. Lines represent mean and colored area SEM. For both panels A and B, # indicates difference between groups.

3.2 Circadian PER2::LUC rhythm

No data were obtained from a number of SCN and liver samples due to low expression of the PER2::LUC protein. In total PER2 expression data were obtained from SCN samples of 8 control and 8 socially defeated animals and from liver tissue of 8 control and 7 socially defeated animals. Panels A and B from Figure 4 show the averages of detrended normalized traces from bioluminescence rhythms in SCN and liver tissue, with the lowest and highest value in each sample trace corresponding to 0 and 100, respectively. Panels C and D show the average phase of the rhythm of PER2::LUC activity from the SCN and liver cultures. In the SCN samples, neither period nor phase were affected by prior social defeat stress (Student's t-test for period: $t(14) = 0.42$, $p = 0.67$; for phase: $t(14) = 0.06$, $p = 0.95$). For the liver samples, the test did not indicate a difference in period ($t(13) = 0.42$, $p = 0.68$), but it did show a significant difference in the phase of the PER2 rhythm, which was delayed by about 8 h in the social defeated group (57.52 h ± 3.17) compared to the control group (49.44 h ± 3.16 h) ($t(13) = 4.93$, $p < 0.01$).

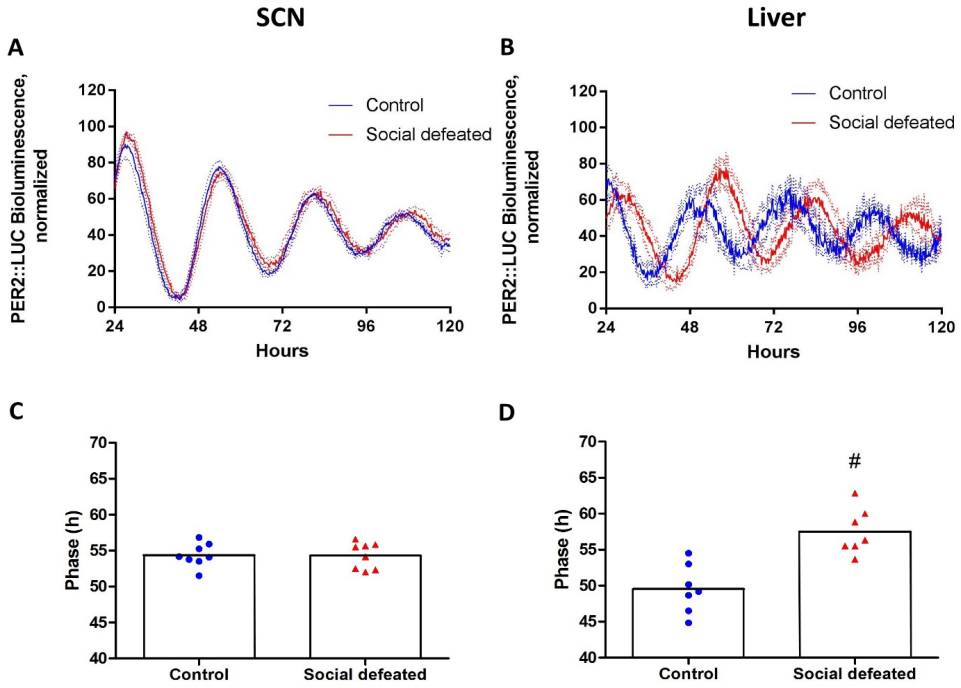

Figure 4. PER2::LUC rhythm in the SCN and Liver tissues collected from social defeated and control mice. Panels A and B) Normalized recording traces with subtracted baselines from SCN and liver slices, respectively. Lines represent mean and dotted lines SEM. Panels C and D) There was no difference between groups for the SCN, but phase was delayed for the Social defeated group in liver cultures. Bars represent mean and symbols represent each individual animal. In panel D, # indicates difference between groups.

4. Discussion

The present results confirmed that daily social defeat stress in mice for 10 consecutive days does not affect the free-running period or phase of the activity rhythm under constant conditions. In agreement with this was the finding that the rhythm of PER2 in the SCN collected after the stress period was unaffected by defeat. In contrast, the phase of the PER2 rhythm in liver tissue was significantly delayed (~6 h) in the defeated as compared to control mice.

Lesioning the SCN results in an arrhythmic activity pattern, indicating that this function is a direct output of the hypothalamic master clock (Richter, 1967; Stephan & Zucker 1972). Both the activity data and the in vitro PER2 rhythm data thus indicate that the SCN is not disturbed by repeated stress. This finding is in agreement with previously published work in rodents subjected to a wide variety of different stressors (Richter, 1967; Meerlo et al. 2002). Our own previous work in rats had shown that social defeat stress either in the active phase or in the rest phase does not affect the phase and period of the free-running activity and temperature rhythms (Meerlo et al. 1997, Meerlo and Daan 1998). More recently, we showed in mice that even daily defeat stress for 10-consecutive days had no effect on the period and phase of the locomotor activity rhythm under constant conditions (Ota et al., 2018, see Chapter 3).

Another recent study on the consequences of chronic intermittent social stress in mice reported small changes in the circadian period and phase of the activity rhythm, in apparent contrast to our current findings (Bartlang et al, 2015). Mice from two different strains (C57BL/6J and C57BL/6N) were subjected to the stress of a social conflict for 19 consecutive days, either in the light or in the dark phase, after which they were maintained in constant darkness to assess the free-running activity rhythms. The analysis suggested a stress-induced delay in the peak of the activity rhythm in both strains, especially when the animals were defeated in the dark phase. A small, but significant shortening of the free-running period by about 10 min was also reported only in the C57BL/6N mice (Bartlang et al., 2015). As discussed by the authors, the apparent phase delays might be explained by an altered rhythm shape rather than a true shift, perhaps as a result of conditioned fear-induced suppression of activity. Since we also observed activity suppression at certain circadian times, we opted to use the activity rise as a more robust phase marker, instead of the peak of activity, which might explain the different findings between the studies. In the experiments by Bartlang and colleagues, repeated defeats stress resulted in a small, albeit significant, 10-min shortening of the circadian period in the C57BL/6N strain. It is unclear why this result was strain-dependent, but the lack of a stress effect in the C57BL/6J mice is in line with the results from our own experiments that were performed in C57BL/6J mice (this study and Ota et al. 2018). Interestingly, another study by the Bartlang group showed that the rhythm of PERIOD2 in the SCN was not affected by their protocol of chronic intermittent defeat stress (Bartlang et al., 2014). This lack of stress effect on clock gene expression in

the SCN is in agreement with the present study, showing no effect of repeated social defeat stress on the SCN PERIOD2 rhythm in vitro. Together, these findings add to the general picture that the endogenous circadian pacemaker is highly resistant to stress.

In contrast to the master clock in the SCN, the liver responded to repeated social defeat stress. The phase of PER2::LUC rhythm was delayed in the liver tissue of defeated compared to control mice. Other studies have also reported phase shifts in clock gene expression in peripheral tissues, including a phase advance in the expression of this clock gene in the adrenal glands of mice defeated during the light phase (Bartlang et al., 2014). Furthermore, restraint stress for 2 h for 3 days in the light phase causes phase advance in the expression of PER2::LUC protein in several tissues in mice, including the liver, whereas it has no effect in the SCN. The same effect is observed with mRNA expression of Per1, Per2, Dbp, and Rev-erbα in the kidney and Per1 and Per2 expression in the hippocampus and cortex (Tahara et al., 2015).

The mechanism through which different stressors affect endogenous clocks in peripheral organs such as the liver, may involve multiple systems and pathways. Stress is a complex phenomenon associated with increased activity of a myriad of neuronal and neuroendocrine systems. Potential candidates for the stress effects on peripheral clocks are the hormones produced by the classical neuroendocrine stress systems, the Hypothalamic-Pituitary-Adrenal (HPA) axis and the Sympatho-Adrenal Medullary (SAM) system. Indeed, Tahara and colleagues (2015) observed a phase advance on the peak of PER2::LUC rhythm in peripheral tissues after exposure to the synthetic glucocorticoid dexamethasone or epinephrine, at ZT4 for 3 consecutive days. Also, it was previously reported that dexamethasone injections can phase shifts clock gene expression in liver, kidney, and heart tissue, but does not affect clock gene expression in the SCN (Balsalobre et al. 2000). These findings suggest that the effects of stress on peripheral clocks may be driven by glucocorticoid hormones, and it is in agreement to the observations that the adult SCN does not express glucocorticoid receptor (Rosenfeld et al., 1988). Nevertheless, more studies are necessary to elucidate whether indeed the effects of chronic social stress on peripheral clocks are directly mediated by glucocorticoid hormones.

In conclusion, chronic social defeat stress did not disrupt the circadian rhythm in the SCN, but it does affect peripheral oscillations in the liver, supporting the idea that disturbances in internal synchronization might be involved in stress-related disorders.

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Chapter 5

Corticosterone delays the phase of PERIOD2 circadian expression in cultured liver tissue

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This chapter is part of a manuscript in preparation to be submitted for publication

Abstract

In a series of previous experiments in mice we found that chronic intermittent social defeat stress does not appear to affect the circadian master clock in the suprachiasmatic nucleus (SCN) but does have an influence on the peripheral liver clock. Specifically, repeated social defeat during the active phase resulted in a robust phase delay of the circadian expression of the clock protein PERIOD2 (PER2) in the liver. One mechanism by which stress might directly affect the liver clock while leaving the master clock in the SCN unaffected is the release of glucocorticoid hormones. Glucocorticoid receptors are abundantly present in liver tissue but not in the adult SCN. To test this hypothesis, we examined whether direct application of corticosterone would alter the rhythm of PER2 expression in isolated liver and SCN samples in vitro. Liver tissue and brain slices containing the SCN were collected from transgenic PERIOD2::LUCIFERASE (PER2::LUC) mice allowing us to track PER2 expression in vitro. The samples were kept in medium containing different doses of corticosterone. As expected, corticosterone did not affect the phase or period of the PER2::LUC protein rhythm of the SCN samples. In contrast, corticosterone caused a phase shift in PER2 in the liver samples. This study confirms earlier findings showing that the SCN seems resistant to stress, but it also shows that clocks in other tissues such as the liver can be affected by stress. Such effects of stress on peripheral circadian oscillators are likely the result of a direct effect of glucocorticoid hormones released from the adrenal gland.

1. Introduction

In mammals, 24h rhythms in physiology and behavior are the result of interacting endogenous clocks that reside in many tissues and organs. This circadian system of clocks allows for the optimal timing of physiological processes among each other and also for an optimal timing of behavior in relation to the day-night cycle in the environment. The endogenous rhythms produced in different tissues and organs are coordinated by a master clock that is located in the hypothalamic suprachiasmatic nucleus (SCN). This master clock in SCN receives direct light input from the retina, which allows it to synchronize the circadian system to the environmental light-dark cycle (Dibner et al., 2010, Saper et al. 2013).

It is generally thought that disturbance of the circadian system and disruption of the normal coordination between internal rhythms can have a negative impact on performance, well-being and health. In the long run, circadian dysfunction might contribute to the development of diseases such as cardiovascular diseases, metabolic syndrome and psychiatric disorders (Baron and Reid, 2014).

Stress is thought to be factor that can affect circadian function and, eventually, contribute to the development of stress-related disorders by a change in circadian organization. This thought is partly based on studies showing that stress-related disorders are often associated with altered rhythms in physiology and behavior, including, for example, changes in hormone rhythms and disturbances of the sleep-wake rhythm (Meerlo et al. 2002).

Collectively, the literature suggests that the master clock in the SCN is highly resistant to stress and stress hormones (Meerlo et al. 2002, Richter 1967). However, recent data suggest that at least some other circadian clocks in peripheral tissues can be affected by stress.

In a series of recent experiments in mice, we found that chronic intermittent social defeat stress does not appear to affect the circadian master clock in the SCN but it does have an influence on the peripheral clock in the liver (see previous chapters). Specifically, repeated social defeat during the active phase resulted in a robust lengthening of the circadian period of the endogenous rhythm in liver PER2 clock protein expression. Likewise, other groups have shown that chronic social defeat shifted the rhythm of PER2 in the adrenal gland, but not in the SCN (Bartlang et al. 2014).

One possible mechanism through which stress might directly affect peripheral clocks such as in the liver while leaving the master clock in the SCN unaffected, is the release of glucocorticoid hormones. The expression of some clock genes can be modulated by glucocorticoids, by means of the binding of the glucocorticoid receptor (GR) to a glucocorticoid response element (GRE) in the promoter region of these genes (Segall and Amir, 2010). Importantly, GR are abundantly present in many tissues and organs, including the liver, but not in the adult SCN (Rosenfeld et al., 1988). The latter might be one explanation as to why the master clock in the SCN appears to be largely resistant to stress.

To test this hypothesis, in the present study we examined whether direct application of the mouse adrenal glucocorticoid corticosterone (CORT) alters the rhythm of PER2 expression in isolated liver and SCN samples *in vitro*. Liver tissue and brain slices containing the SCN were collected from transgenic PER2::LUC mice allowing us to track PER2 protein rhythm *in vitro*. The liver and SCN samples were exposed *in vitro* to CORT.

2. Material and Methods

2.1 Animals and housing

Tissues from 20 male PER2::LUC knock-in mice with a C57BL/6 background (Yoo et al. 2004) from our own colony were used in this experiment. The animals were maintained in a room with controlled temperature and 12:12 LD cycle, housed in groups of maximum 4 per cage.

2.2 Experimental design

The mice in this experiment were maintained in a room with controlled temperature and 12:12 LD cycle, housed in groups of maximum 4 per cage. The animals remained undisturbed until the moment of tissue collection. Half of the animals was killed at ZT 11 and the other half at ZT 23. Liver samples and brain sections containing the SCN were directly exposed to CORT in vitro. Although we aimed to assess the effect of chronically elevated CORT levels, we collected tissues at 2 different time points to determine whether or not the starting time of the treatment in itself could have an effect.

2.3 Tissue collection and processing

The procedures for tissue preparation and in vitro recording of PER2::LUC activity were done with minor adaptations from the protocol of Yamazaki and Takahashi (2005). The animals were euthanized by decapitation at ZT 11 or ZT 23. From each mouse, six liver samples were taken and cultured in separate dishes with pre-warmed recording medium with a high, medium or zero concentration of CORT (duplicates for each concentration). Coronal brain slices (200 µm) were prepared on a vibratome (CI.7000SMZ, Campden Instruments Ltd., Leicester, U.K.) in chilled Hanks' buffered salt solution (HBSS), after which slides containing the SCN were selected and the SCN's from the left and right hemisphere were separated from each other and from the surrounding brain tissue using a scalpel. The left and right SCN samples were placed separately in dishes with culture plate inserts and pre-warmed recording medium. Because of the small size of the SCN and the limited number of sections containing this nucleus (1-2), SCN samples were only exposed to the zero and high concentration of CORT (if possible duplicates). For each mouse, the SCN from one hemisphere was placed in medium containing the high concentration of corticosterone, while the SCN from the other hemisphere served as control and was placed in medium without CORT. The dishes with the samples were placed in the recording apparatus LumiCycle

32 (LumiCycle, Actimetrics Inc., Evanston, IL) and bioluminescent activity was recorded for 5 days at 10 minute intervals, at 37°C.

2.4 Corticosterone concentrations

The medium used in the present study was the same as published standards (Yamazaki and Takahashi, 2005), except that the B27 supplement was substituted by modified NS21 (Crosby et al., 2017) without CORT, which was added in different concentrations. CORT was dissolved in ethanol and later diluted in recording medium, aimed at final CORT concentrations of around 300 ng/ml (medium physiological concentration) and 900 ng/ml (high concentration). Due to ethanol volatility, the final concentrations were somewhat higher and ranged from 374.5 ng/ml to 556.5 ng/ml (medium-high) and 877 ng/ml to 1577.5 ng/ml (high), respectively. A pilot study was performed to assess if and how much the concentration of corticosterone added at the start of the recording would change over the 1 week recording period. Samples from the recording medium with and without liver tissue were collected at different time intervals and analyzed for CORT by radioimmunoassay (MP Biomedicals, Eschwege, Germany).

2.5 Data analysis

Data were analyzed from hour 36 to 120 (hour 0 corresponded to the start of bioluminescence recording). The first 24 h were excluded because the cellular bioluminescence during this time may not reflect PER2 rhythmicity, and instead, may be related to dissection and media change. The analysis started at hour 36 due to the method used to remove drift in the bioluminescence level. The drift in the recording trace was removed (detrended) using a 24 h moving average. The detrended data were analyzed in GraphPad Prism (version 7.00 for Windows, GraphPad Software, La Jolla California USA) by fitting a cosine wave, accounting for damping of oscillatory amplitude, described by Crosby and colleagues (2017). The period of the fitted cosine was considered the period of the PER2 rhythm. The phase was determined by selecting the second peak of PER2::LUC rhythm in each sample. When both liver samples of the same CORT concentration for a given animal survived, the results of these two samples were averaged. A Student's t-test was used to analyze the effects of CORT on period and phase of the PER2::LUC rhythm.

3. Results

Our pilot study showed that the CORT concentrations in the medium remained stable over a one-week recording period (Figure 1). This was true for both the medium and high CORT concentrations.

Although CORT concentrations were stable across the 7-day recording period, the onset of CORT exposure differed, such that samples from half of the mice were collected and exposed to CORT starting at ZT 11 (towards the end of the normal resting phase of the

mouse) and samples from the other half of the mice were collected and exposed to CORT starting at ZT 23 (towards the end of the normal active phase of the mouse).

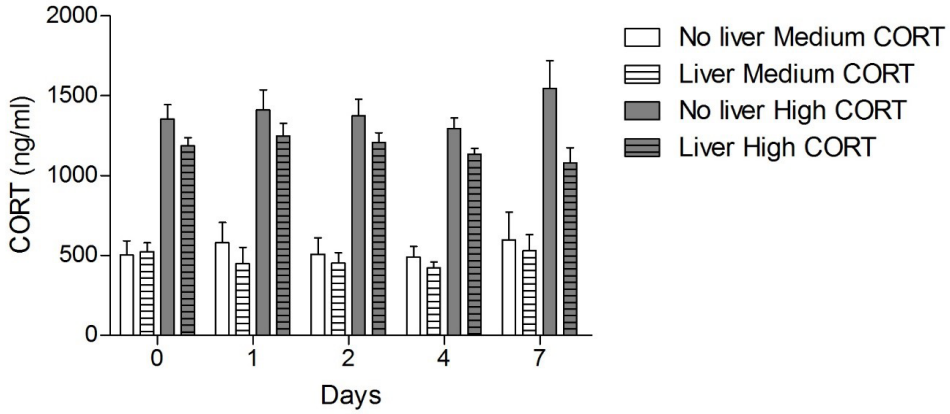

Figure 1. Corticosterone concentration along the days. Corticosterone concentration in the medium of plates with and without liver tissue before closing the dish, within 1, 2, 4 and 7 days in the LumiCycle. Corticosterone levels seem to remain stable during the 7 days of recording.

Not all tissue samples survived the preparation procedure and expressed sufficient levels of PER2. For samples collected at ZT 11 (Figure 2), the data correspond to samples of SCN exposed to high CORT (4) or to no CORT (4) in the medium. A total of 6 successful measurements for each concentration were obtained for liver samples exposed to high, medium, or no CORT in the medium. For the samples collected at ZT 23 (Figure 3), the data correspond to 8 samples of SCN exposed to high concentrations of CORT and 8 to no CORT. A total of 6, 5 and 6 successful measurements were achieved of liver samples exposed to high or medium CORT concentrations or no CORT, respectively.

Panels A and B of figures 2 and 3 show normalized and averaged traces acquired from bioluminescence recordings of the SCN and liver tissues, respectively. Because of the dampening of the rhythm, data were analyzed from days 1 to 5. The amplitude variation of the rhythm varied among samples, and therefore this aspect was not analyzed.

Figure 2 shows the effects of CORT at ZT 11 on the phase of PER2::LUC rhythm. The Student's t-test did not reveal an effect of CORT on period ($t(6) = 1.79$, $p = 0.12$) or phase ($t(6) = 1.33$, $p = 0.23$) in the SCN. ANOVA did not show an effect of CORT on period ($F(2,15) = 0.06$, $p = 0.95$) but revealed an effect on phase ($F(2,15) = 4.25$, $p = 0.03$) in the liver. The post-hoc analysis showed a delayed phase of the second peak of the PER2::LUC rhythm in medium CORT concentration (54.99 h ± 1.23 h) compared to no CORT (51.04 h ± 1.17 h)($p = 0.04$), and a trend for effect of high concentration (54.58 h ± 0.68 h) compared to No CORT group ($p = 0.07$).

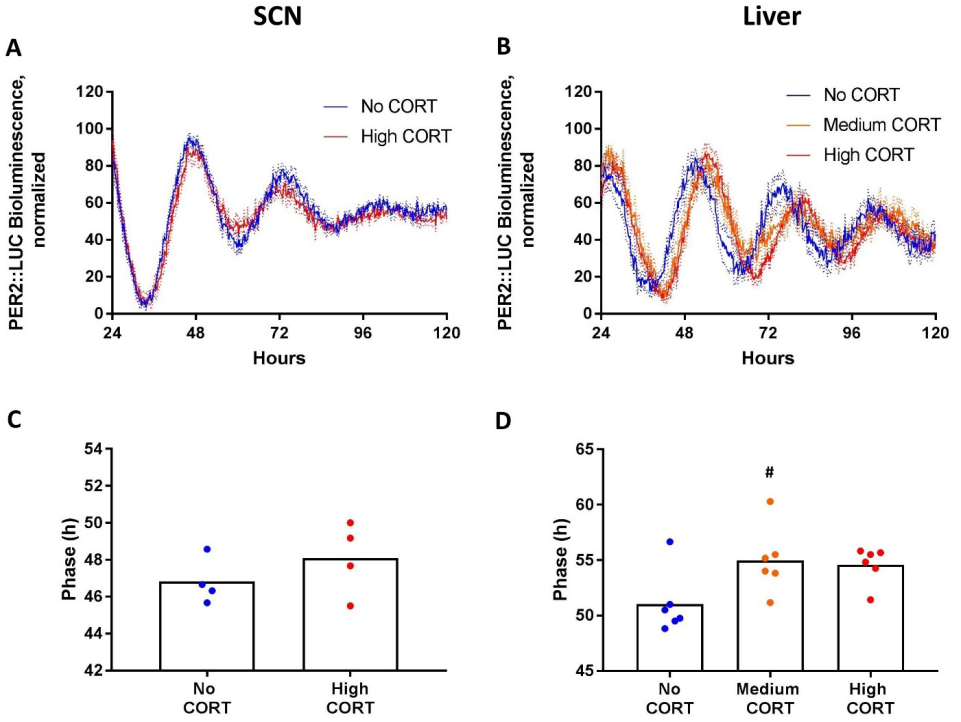

Figure 2. Effects of corticosterone on phase of PER2::LUC rhythm in tissues collected at ZT 11. Panels A) and B) Normalized recording traces with subtracted baseline from SCN and liver tissues, respectively. Lines represent mean and dotted lines SEM. C) There was no significant effect of corticosterone on circadian phase in the SCN. D) Medium concentration of corticosterone delayed circadian phase in the liver. In panels C and D, bars represent mean and symbols represent each individual animal. # Indicates difference from No CORT group.

Figure 3 illustrates the effects of corticosterone at ZT 23 on phase of PER2::LUC rhythm. There was no effect on period ($t(14) = 0.015$, $p = 0.99$) or phase ($t(14) = 1.09$, $p = 0.29$) in the SCN. ANOVA showed a trend for effect on period ($F(2,14) = 2.95$, $p = 0.085$) but no effect on phase ($F(2,14) = 1.78$, $p = 0.20$) in the liver.

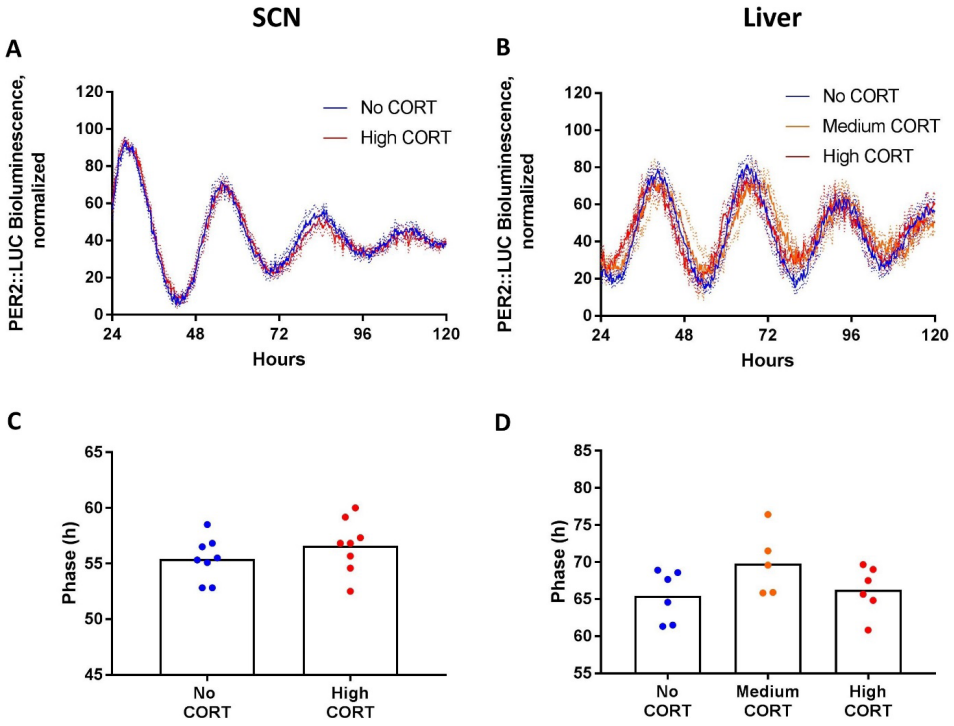

Figure 3. Effects of corticosterone on phase of PER2::LUC rhythm in tissues collected at ZT 23. Panels A and B) Normalized recording traces with subtracted baseline from SCN and liver tissues, respectively. Lines represent mean and dotted lines SEM. C) There was no significant effect of corticosterone on SCN phase. D) There was no significant effect of corticosterone on circadian phase in the liver. In panels C and D, bars represent mean and symbols represent each individual animal.

4. Discussion

The present data collected from isolated liver and SCN tissue in vitro confirm the hypothesis that glucocorticoids can directly affect the peripheral liver clock but not the master clock in the SCN. We found a phase shift of the circadian period in PER2 rhythm of liver tissue, similar to what we previously showed in liver tissue collected from animals that had been exposed to chronic intermittent social defeat stress (Chapter 4). These results suggest that the effects of chronic social stress on peripheral oscillators may be mediated by direct actions of glucocorticoids and they also corroborate previous findings that the master oscillator is not susceptible to stress.

Other works with treatment *in vitro* have also reported direct effect of glucocorticoids on PER2 rhythm. In the nasal mucosa tissue of PER2::LUC mice, dexamethasone caused a phase maximum advance when administered at CT 18 and a maximum phase delay when administered at CT 12 (Honma et al., 2015). Embryonic fibroblasts from PER2::LUC knock-in mice treated with dexamethasone had an increase in PER2 protein levels and a phase delay of the gene expression rhythm, and when the cells were treated with a GR antagonist, these effects were blocked, showing that the glucocorticoid effect is dependent of this receptor (Cheon et al., 2013).

Our findings are also in agreement with a study by Tahara and colleagues (2015), who observed a phase advance of the peak of the PER2::LUC rhythm in peripheral tissues after exposure to the synthetic glucocorticoid dexamethasone, epinephrine, at ZT 4 for 3 consecutive days. Also, it was previously reported that dexamethasone injections can phase shifts clock gene expression in liver, kidney, and heart tissue, but does not affect clock gene expression in the SCN (Balsalobre et al. 2000). In humans, treatment with Cortef (a synthetic hydrocortisone) for 6 days phase shifted PER2–3 and BMAL1 rhythm in peripheral blood mononuclear cells, but neither phase nor the amplitude of plasma melatonin rhythm were modified, indicating that the central clock was not affected by the glucocorticoid (Cuesta et al., 2015).

Similarly to other studies using glucocorticoid treatments, and to our own results with social stress, we did not observe an effect of CORT on PER2 rhythm in the SCN tissue. Interestingly, a 6-week treatment of adrenalectomized mice with hydrocortisone in drinking water phase shifted Per1-LUC expression in different peripheral tissues, caused phase desynchrony in the liver and also advanced phase in the SCN (Pezük et al., 2012). The authors discussed that glucocorticoids could affect the master clock by disturbing the raphe nuclei, which sends inputs to the SCN. Although our previous studies did not indicate an effect of 10 days of social stress on the SCN, perhaps a more prolonged stress could have this effect in the living animal.

The present data collected from isolated liver and SCN tissue in vitro confirmed the hypothesis that glucocorticoids can directly affect the peripheral liver clock but not the master clock in the SCN. These results suggest that the effects of chronic social stress on peripheral oscillators may be mediated by direct actions of glucocorticoids and they also corroborate previous findings that the master oscillator is not susceptible to stress. Together with previous literature, our results suggest that the effects of stress on peripheral clocks may be driven by glucocorticoid hormones, and it is in agreement to the observations that the adult SCN does not express the glucocorticoid receptor (Rosenfeld et al., 1988).

In conclusion, our experiments show that chronic social stress does not disturb the master clock in the SCN, but it that it is likely to shift circadian phase in the liver through elevated of corticosterone levels. This finding shows a mechanism by which stress leads to the often-observed circadian disruptions in stress-related diseases.

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Chapter 6

Continuous social defeat for ten days did not induce permanent behavioral alterations in adult mice

Simone Marie Ota, Peter Meerlo, Deborah Suchecki

Abstract

Social stress has been associated with development of many psychiatric disorders, such as anxiety and depression. Social defeat stress has been used as a model to induce depressive-like behavior in animals. As shown in our previous studies, mice exposed to social defeat stress present suppression of locomotor activity. But this suppression is mostly observed during the days that stress is applied and is not persistent after some days of recovery. Therefore, the aim of this study was to observe whether chronic social defeat stress would have persistent on social investigation behavior and changes in sucrose preference over time in adult mice. For this purpose, C57BL/6 male mice were used as experimental animals and Swiss mice were used as aggressors. The social defeated group was submitted to 10 days of continuous social defeat while the control group cohabitated the cage with other mice from the same line, without physical contact. Four days after the defeat phase, social investigation was observed. Sucrose preference and weight were assessed during habituation, social defeat and recovery. There was no significant effect of social defeat neither in sucrose preference nor in social investigation compared to control group. However, during the social defeat phase, the animals in general presented lower sucrose preference compared to the other phases. Curiously, the social defeat group gained weight throughout the experiment, but the control group did not. In conclusion, continuous social defeat for 10 days did not cause permanent changes on behavior in adult mice, although manipulations may have caused stress on the control group.

1. Introduction

Stressful events, such as job loss, divorce and widowhood, have been reported to trigger the development of depression (Mandal et al., 2011; Aseltine and Kessler, 1993; Umberson et al., 1992, respectively). Furthermore, chronic rather than acute stress seems to be a stronger predictor (Mcgonagle and Kessler, 1990) as several studies report associations between chronic work stress and depression (Wang and Patten., 2001; Lunau et al., 2013; Gherardi-Donato et al., 2015; Hoven et al., 2015). Griffiths and colleagues (2014) observed that defeat (understood as failed social struggle) and entrapment (inability to escape from a situation) predict increased levels of depression and state anxiety 12 months later, irrespective of initial symptoms levels. Despite the evidence, it is still difficult to establish causal relationships.

To study the relationship and try to determine the causal role between chronic social stress and depression, animal models, such as the social defeat, have been used. Chronic social stress is an important risk factor for the development of depression in humans (Johnson and Sarason, 1978; Kessler, 1997; Hammen, 2005). In general, exposure to social defeat stress induces depressive-like behaviors such as reduced social interaction (Favoretto et al.,2017, Krishnan et al., 2007, Macedo et al., 2018, Meerlo et al, 1996; Venzala et al, 2012), decreased weight gain (Carnevali et al, 2012; Meerlo et al, 1996; Venzala et al, 2012) and lower sucrose preference (Favoretto et al.,2017, Krishnan et al., 2007, Macedo et al., 2018, Miczek et al, 2011; Venzala et al, 2012). This model is based on the resident-intruder paradigm and simulates episodes of social stress. The resident is trained to display aggressive behavior and it is expected that it becomes the dominant and the intruder becomes subordinate, to emulate the social hierarchy and subordination seen in some human conditions (Koolhaas et al., 1997). However, many studies with chronic stress in mice are focused on the effects in adolescents and behavioral tests are conducted right after stress procedures. In our studies, we have observed that intermittent social stress for 10 days suppressed locomotor activity in mice during stress days (Ota et al., 2018, Ota et al. – in preparation, see chapters 3 and 4). Therefore, in this study, we investigated whether chronic social stress for 10 days could induce persistent depressive-like behavior, not only during or acutely after defeat, but also after some days of recovery, in adult C57BL/6 mice.

2. Material and methods

2.1 Animals and housing

Fourteen three-month-old C57BL/6 male mice were used as experimental animals and eleven five-month-old Swiss mice, previously trained, were used as aggressors. The animals were obtained from Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia (CEDEME) of Universidade Federal de São Paulo. All animals were maintained in 12:12 light/dark cycle and controlled temperature (23 ± 2° C), with free access to water and food.

2.2 Experimental procedure

The timeline of the experiment consisted of three blocks of 10 days: Habituation, Social Defeat and Recovery, as shown in Figure 1. The animals were divided into Control or Social Defeated group, based on their body weight and sucrose preference. Weighing occurred before the sucrose preference test, three times during each block, every four days. The mean of the three measurements was calculated for each phase of the protocol. During Habituation, the animals were left in their cages, except for weighing. During the Social Defeat block, mice were placed in a new cage, either with an aggressor (Social defeated group) or a naïve mouse (Control group), as described below. Then, 24 h after the last defeat, the animals returned to their own home cage and were left undisturbed until testing in the Recovery block.

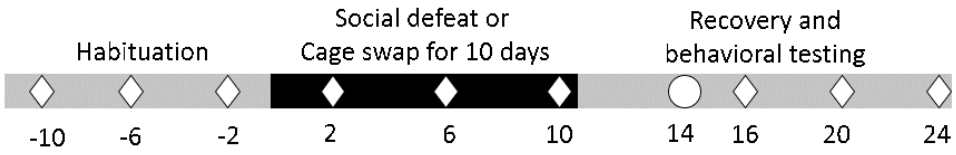

Figure 1. Timeline of the experiment. Diamond represents sucrose preference test and weighing, circle represents social investigation test.

2.3 Social Defeat

Social defeat stress was conducted similarly to that proposed by Golden and colleagues (2011). The intruder mice (Social Defeated group) were placed in the aggressor's home cage with a perforated Plexiglas divider between them, which allowed olfactory and visual, but no physical, contact. After five minutes, the partition was removed, and the resident threatened and attacked the intruder. This phase also lasted five minutes, after which the partition was replaced, and the intruder remained in the resident´s cage until the next day, when it was exposed to another aggressive animal. This procedure was repeated throughout the 10 days of social defeat. The animals in the Control group were kept in similar cages, together with a naïve animal of the same strain and age but separated by a divider to avoid physical contact. Control animals were also exposed to a new cage mate throughout the 10 days of the protocol.

2.4 Sucrose Preference

Before onset of the experimental procedure, the animals were presented with two drinking bottles, one containing a 1% sucrose solution and another containing water, for 72 h, to stimulate sucrose intake and balancing of sucrose preference between groups. On testing days, which occurred every four days, the mice were placed back in their own cage for 1 h (at ZT 6) and the two bottles were presented again. The intake in each block (Habituation, Social Defeat and Recovery) was calculated as the mean of three tests. For every test, the position of the bottles was alternated to avoid preference.

2.5 Social Investigation

The animals were placed in the social investigation arena for ten minutes the day before the test for habituation. In the test day, animals were placed in the middle of the arena, with a cage with a naïve animal (same strain and age) and an empty one in opposite sides. Number and time of investigation of each cage were observed and the percentages (investigation of cage with naïve/ investigation of cage with naïve + investigation of empty cage) compared between groups.

2.6 Data Analysis

Data from body weight and sucrose consumption were analyzed with a two-way ANOVA for repeated measures, with the between-subjects factor GROUP (Control x Social Defeated) and within-subjects factor TIME (Habituation, Social Defeat, Recovery). The Newman-Keuls test was used as a *post-hoc* test for the sucrose consumption data. Social investigation data were analyzed with Student´s t test independently for each parameter evaluated. For all statistical tests, the level of significance was considered as $p \leq 0.05$.

3. Results

3.1 Body weight

The animals were weighted every four days, and because there was no difference among the days in each block, we averaged the data for each block. Statistical analysis showed an effect of time ($F_{2,24}$= 15.60, $p < 0.01$) and interaction between factors TIME and GROUP ($F_{2,24}$=7.47, $p < 0.01$). The *post-hoc* analysis revealed that the Social Defeated group was heavier during defeat ($p < 0.03$) and Recovery ($p < 0.01$) compared to Habituation and it was also heavier during recovery than during Social Defeat ($p < 0.01$).

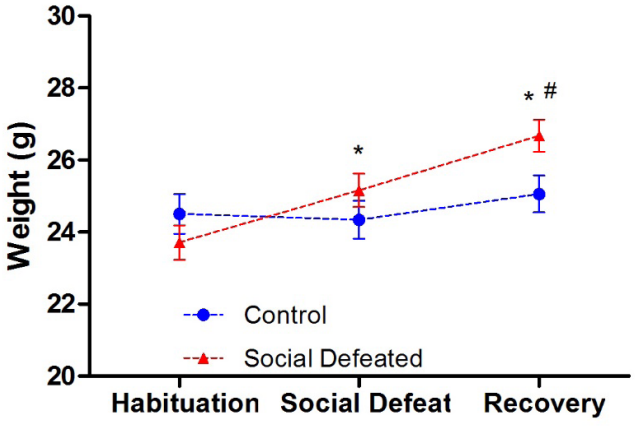

Figure 2. Body weight. Social defeated animals gained weight during the experiment, whereas the control animals did not. The results are represented by mean ± s.e.m. * Different from HAB, # different from SD.

3.2 Sucrose Preference Test

The animals were tested for sucrose preference three times during Habituation, Social Defeat and Recovery days, but since there was no difference among the days in each block, we averaged the three measurements per block. The two-way ANOVA for repeated measures showed an effect of TIME ($F_{2,24}$ = 4.88, p = 0.02). The Newman-Keuls test revealed that during the Social Defeat block, the animals in general presented lower sucrose preference compared to Habituation (p = 0.02) and Recovery (p = 0.03) blocks.

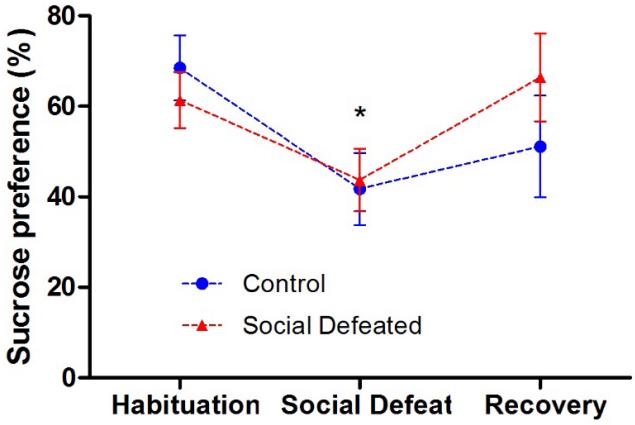

Figure 3: Sucrose preference. In general, the animals displayed lower sucrose preference during period of social defeat compared to the other periods. The results are represented by mean ± s.e.m. *Different from Habituation and Recovery.

3.3 Social Investigation Test

Student´s t-test revealed no significant difference between groups in the percentage frequency of investigation for Control (46.15% ± 10.53%) and Social Defeated (56.79% ± 10.50%); (t(12) = 1.87 , p=0.08). No significant difference was observed in the percentage of time of investigation between Control (53.67% ± 14.30%) and Social Defeated (62.38% ± 9.01%); t(12) = 1.40 , p = 0.18.

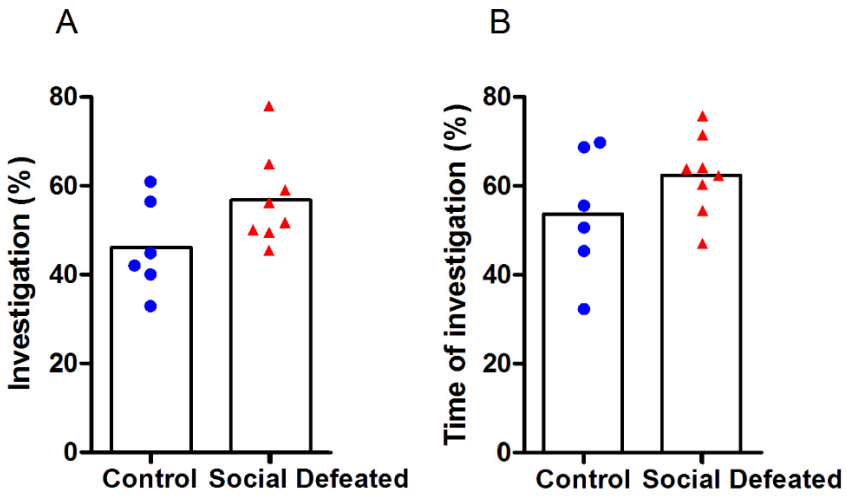

Figure 4. Social Investigation. Panel A) There was no significant difference in frequency of investigation between groups. Panel B) The test did not show difference in investigation time between groups. The bars represent mean.

4. Discussion

The results of the present study showed that continuous social defeat for 10 days in adult mice did not produce significant alterations in behavior compared to control animals exposed to an unfamiliar cage mate without physical contact. This finding contradicts what is found in the literature. Chronic social defeat is reported to induce anxiety and depressive-like behavior in rats (Carnevali et al, 2012; Meerlo et al, 1996; Miczek et al., 2011) and mice (Krishnan et al., 2007; Venzala et al., 2012). One possible explanation for the unexpected results is the small number of animals in our study and large intragroup variation. Besides, Golden and collaborators (2011) reported a 30-40% rate of animals resilient to the social defeat. However, observing the distribution of frequency and time of social investigation in our study, the control group seemed to investigate the naïve animal less, although there was no significant difference between the groups. Furthermore, the control group did not gain weight during the experiment, indicating that this group might have also been stressed by the manipulation.

Some differences between ours and other protocols may explain the lack of social defeat effects. In this study, defeat started when animals were fully adults (three-month old), and although studies in rats show effects of defeat on social exploration (Meerlo et al., 1996), Golden and collaborators (2011) state that the negative effects are more pronounced when mice are defeated during early adulthood (7-8 weeks). We do not believe that the strain of the aggressive animal (Swiss, not CD1) had any influence on the results, since they were trained to reach high scores of aggression. On the other hand, control animals were

paired with age matched mice, and that 12-week old mice present more aggressive behavior than 8-week olds (Kawai et al., 2003). Therefore, even when physically separated, control mice might have been exposed to visual and auditory aggressive displays by their cage mates. Furthermore, control animals might also have been stressed because the rotation of cage mates caused social instability. It has been shown that rats from different cages, when housed together, present higher levels of ACTH and corticosterone than rats from social stable groups (Suchecki and Tufik, 2000). Lower body weight and worse fur condition was observed by Boleij and colleagues (2014) in response to changes in group composition twice a week for seven weeks. It was hypothesized by Meshalkina and Kalluef (2016) that mice from the same strain, more similar in size could fight more fiercely to determine the winner, leading to a stronger social stress. If this is the case, our control mice could have experienced social stress, although there was no experimentally induced physical interaction during the defeat phase for the animals in the control group.

The results observed for sucrose preference may be due to the already low level of preference in the beginning of the experiment. Krishnan and colleagues (2007) observed that sucrose preference for controls was higher than 80%, while in our study the preference was around 64% for both groups before the onset of social defeat and dropped to 43% during the stress period. Nevertheless, decrease in the sucrose intake after maintaining the control mice with another individual in the same cage was also observed in another study (Venzala et al., 2012). One might argue that single housing could induce depressive-like behaviors (Chourbaji et al., 2005). However, in our study, mice were already individualized in the habituation phase and the reduction in sucrose consumption occurred during the social defeat phase, without difference of intake between recovery and habituation phase, indicating that social defeat and housing with an unknown mouse induced this depressive-like behavior, with no permanent effect after the end of stress.

Regarding the social investigation test, some differences are observed in our protocol compared to other studies. In our protocol, the animals were habituated to the empty arena one day before and on the following day, they were placed in the arena with an empty cage and one cage containing the naïve target animal at the same time, while in other studies, the animals were exposed to the arena with an empty cage and some minutes later, the naïve animal was introduced (Favoretto et al., 2017, Golden et al., 2011, Krishnan et al., 2007, Macedo et al., 2018). In the studies from Krishnan and colleagues (2007) and Golden and colleagues (2011), the naïve animal was from the same strain as the aggressors (CD1), whereas in our study, the naïve animal was from the same strain as the intruder (C57BL/6). The question of whether the use of the same strain as the aggressor for the target mouse could affect the results was also discussed by Meshlakina and Kalluef (2016). They argue that the use of the “heavier winner” strain could result in a conditioned avoidance rather than a lack of interest for social interaction. On the other hand, Venzala and collaborators (2012) also used a naïve animal from the same line as the intruder and observed less interaction by

the defeated group compared to control animals.

Finally, some studies do not provide neither the time of day when defeats nor behavioral tests start. This detail is relevant once Bartlang and colleagues (2012) showed that social defeat during the night, but not during the day, affects time spent in social contact zone. Furthermore, it was reported that male rats travelled more in the center of the open field during the dark phase than in the light phase, suggesting that evaluation of some behaviors are affected by the time of testing (Verma et al., 2010). However, Verma and colleagues (2010) did not observe differences in sucrose consumption between light and dark phases.

In conclusion, chronic social defeat in the light phase did not induce permanent depressive-like behavior in adult C57BL/6 mice compared to control animals maintained with an unfamiliar mouse in the same cage.

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Chapter 7

General Discussion

The circadian system generates internal time in an organism and synchronizes it to external time, a property that permits anticipation and preparation so that physiological and behavioral processes occur with maximal efficiency. In mammals, the most important signal to reset the master oscillator, located in the SCN, is the light-dark cycle. Although other signals such as food and temperature are able to influence peripheral oscillators, the SCN does not seem to be affected by them. The main aim of this thesis was to evaluate whether chronic social defeat stressor exposure to glucocorticoid stress hormones can perturb the master clock and/or peripheral oscillators.

1.Effects of social defeat stress

We found that in male mice repeated social defeat stress on 10 consecutive days results in a strong suppression of locomotor activity (Chapters 3 and 4), which confirms earlier findings in both mice (Wells et al., 2017) and rats (Meerlo et al., 1997, Meerlo et al., 2002). Activity levels from defeated animals returned to the levels of control animals after a few days of recovery. The strong suppression of activity indicates that one way or another the mice were severely impacted by defeat, which therefore seems to represent a good model to study effects of stress on circadian function.

Although the overall level of locomotor activity was suppressed by social defeat stress, the period and phase of the rhythm as calculated from successive activity onsets did not differ between defeated animals and control mice. These results are in agreement with earlier findings on effects of social defeat stress in rats (Meerlo et al., 1997, Meerlo and Daan, 1998). In our new experiments the mice were subjected to defeat repeatedly for 10 days to assess of this chronic intermittent stress potentially has small cumulative effects that perhaps went unnoticed in the earlier studies in rats that were subjected to defeat stress only once or twice. This was not the case. Even chronic stress did not affect period and phase of the activity rhythm.

In chapters 4 and 5, we used a different approach to assess the effects of stress on the molecular circadian clock in the SCN directly. We used knock-in mice that have the *Luciferase* gene fused to the *Per2* gene, which allowed us to analyze PER2::LUC protein expression, by recording the bioluminescence in tissue cultures (Yoo et al., 2004). The mice were exposed to 10 days of social defeat stress and subsequently the SCN was cultured to assess the circadian rhythm in PER2::LUC expression in vitro. As we hypothesized, the rhythm of PER2::LUC expression was not affected in the SCN, neither by repeated social stress prior to the collection of SCN tissue (Chapter 4) nor by the stress hormone corticosterone added to the recording medium directly (Chapter 5). Again, these findings clearly support the general notion that the master clock in the SCN is well protected against any perturbing influence of stress.

In contrast, our studies showed that the circadian oscillator in the liver was affected by both prior social defeat stress (Chapter 4) and direct exposure to glucocorticoids in vitro

(Chapter 5). The phase of PER2::LUC expression was delayed, both by social defeat and corticosterone treatment at ZT11 on isolated liver tissue. Given that the effects of prior social defeat stress and direct exposure to glucocorticoids on the liver rhythm were at least qualitatively similar, it is likely that the effects of stress are mediated by glucocorticoid hormones. This might also explain why stress appears to have little effect on the master clock in the SCN since, unlike most other tissues and brain regions, the adult SCN does not contain glucocorticoid receptors (Balsalobre et al., 2000, Rosenfeld et al., 1988).

Tahara and colleagues (2015) had already demonstrated that restraint stress for 3 consecutive days was able to phase shift the peak of PER2::LUC expression and RNA expression of other clock genes in different tissues, but not in the SCN of mice under LD. In the same study, social defeat and injection of dexamethasone for 3 days were also able to phase shift PER2 expression in peripheral tissues (liver, kidney and submandibular gland). Balsalobre and colleagues (2000) had previously reported that injections with the synthetic glucocorticoid dexamethasone were able to phase shift clock gene transcription in peripheral tissues but not in the SCN. Moreover, our *in vitro* measurements demonstrate that the effects of glucocorticoids may be directly mediated by binding to glucocorticoid receptor in the liver tissue itself (chapter 5).

In chapter 6, we have described a study to assess whether social defeat stress in mice would result in depressive-like behavior. This study was based on the hypothesis that circadian dysfunction might contribute to disease processes, including the development of psychiatric disorders such as depression. While the earlier chapters clearly demonstrate a very strong defeat stress-induced suppression of general home cage activity, which might be taken as an indicator of depression in itself, the more specific read outs of anxiety and depression in chapter 6 were not affected. This unexpected finding is in contrast with our earlier work in rats, in which the behavioral changes following social defeat stress have been presented as a model of depression (Koolhaas et al., 1997). Based on our mixed findings in mice, it is possible that defeat stress did not truly result in a depression-like state or that the behavioral read outs in chapter 6 were not sufficiently sensitive for mice. The behavioral assessment in chapter 6 started 4 days after the last defeat, when the suppression of home cage shown in chapter 3 had largely recovered and were no longer different from control. Perhaps in our model of social defeat stress in this particular strain of mice, behavioral changes are mostly acute and normalize within a few days after the last stressor. It is not excluded that stronger and more persistent stress-induced behavioral changes may occur under slightly different conditions and/or in mice with different traits. For example, one factor of importance that may have attenuated behavioral changes in chapter 6 is the age of the mice. Golden and colleagues (2011) have suggested that depressive-like behavior is best induced in younger mice. Since we were interested in observing the effects of stress in adulthood, we exposed our mice to defeat after 3 months of age, when the mouse brain is considered to be fully developed (Hammelrath et al., 2016). Future experiments are

required to assess depression-like behavior in more detail and also its potential relationship with changes in circadian function.

In summary, our studies in mice show that sever, uncontrollable social defeat stress does not appear to affect the master clock in the SCN, but it does phase shift the peripheral clock in the liver, an effect that may be mediated by glucocorticoid stress hormones.

2. Role of CORT in stress effects on circadian function

Stress is also associated with an activation of the sympathoadrenal system, resulting in the rapid release of the catecholamines noradrenaline and adrenaline. Tahara and colleagues (2015) also tested the effects of adrenaline and noradrenaline *in vivo* injections and reported that these hormones can also phase shift PER2 peak in the liver, kidney and submandibular gland. Therefore, it is still a question whether effects of stress on the liver clock are mediated by glucocorticoids, catecholamines, or both. Based on our own findings, one logical follow-up study to address this particular issue would be to inhibit the release of corticosterone during each successive social defeat stress by administration of metyrapone, a reversible inhibitor of an enzyme involved in cortisol and corticosterone synthesis. Then, if the phase shifts of the liver clock are no longer observed, one could conclude that corticosterone is necessary for the stress effects on this peripheral oscillator.

Alternatively, to test if effects of stress on peripheral clocks are mediated by catecholamines, a follow-up study could be the administration of catecholamine receptors blockers during the 10-days social defeat stress. Carvedilol, for example, is a β1, β2 and α1 adrenergic receptor blocker used to treat hypertension and congestive heart failure, and it is known to reduce the amplitude of heart rate (Valentina et al., 2015). Another approach could be the inhibition of dopamine beta-hydroxylase, the enzyme that converts dopamine into noradrenaline, during the days of social defeat exposure by administration of nepicastat, although no studies on the circadian effects of this drug were found.

It is possible that some effects of social defeat stress might be mediated by changes in body temperature (Pittendrigh, 1981). Especially circadian rhythms in peripheral tissues are sensitive to temperature changes (Buhr et al. 2010). Like most stressors, defeat is associated with an acute and strong increase in body temperature (Koolhaas et al. 1997). Moreover, defeat stress in particular has been reported to cause rather long-lasting elevations of body temperature, mainly during the circadian resting phase (e.g., Meerlo et al., 1996, Meerlo et al., 1997). However, increased body temperature as well as increased activity caused by cage change stimulation for 3 days does not seem to shift the peak phase of PER2 expression in the liver, kidney and submandibular gland (Tahara et al., 2015). Therefore, stress-induced temperature increases do not seem to be the most plausible explanation for the shift in the liver clock following social defeat.

3. Changes in PER2 rhythm and liver function

The liver plays an important role in the regulation of glucose homeostasis, together with other tissues such as the pancreas. The circadian system in turn provides the rhythmicity in baseline glucose levels in synchrony with the environment and in anticipation to regular periodic events, such as feeding during the active phase and fasting during the inactive phase (Reinke and Asher, 2016). Several hepatic enzymes participating in lipid biosynthesis and catabolism are expressed in a daily manner (e.g. cytochrome P450s, HMGCoA reductase, Lipin), (Panda, 2016) and the liver oscillator appears to buffer excessive circadian fluctuations of glucose levels in the blood caused by behavioral rhythms. Mice with liver-specific deletion of the core gene Bmal1, showed hypoglycemia during the fasting phase, exaggerated glucose clearance, and loss of the circadian expression rhythm of glucose regulatory genes in the liver (Lamia et al., 2008).

Mice with a Per2 knockout are heavier than wild type mice during the pre-adolescence and adolescence phase of life. After this, their growth rate slows down, and they become lighter than the wild types in adulthood. It was shown that Per2 knockout mice have the same daily food intake as wild type mice, but around 50% reduced triglycerides plasma levels (Grimaldi et al., 2010). In the same study, the authors demonstrated that PER2 controls lipid metabolism by repressing peroxisome proliferation activated receptor γ2 (PPARγ2), an important regulator of lipid metabolism and adipocyte differentiation.

These findings together demonstrate that the circadian system and specific clock genes play an important role in metabolic regulation. Moreover, altered circadian function and changes in clock gene expression may in the long run sensitize individuals for metabolic disorders such as diabetes and obesity (Knutsson and Kempe, 2014, Tucker et al., 2012). Our own findings on social defeat-induced phase shifts of the liver clock may imply that stress could be one of the factors leading to disturbed circadian regulation of metabolic function and ultimately metabolic disorders. However, it remains difficult to study the relationship of phase shifts caused by stress with metabolic diseases, since the available techniques to track, for example, PER2 expression involve *in vitro* culture or anesthetizing the animals. Nevertheless, it is an interesting issue, because individuals under chronic stress often have clinical manifestations of metabolic syndrome (Nicolaides et al., 2014).

4. Oscillators in other brain tissues

As discussed above, circadian disruption between peripheral oscillators, such as the liver, might implicate in metabolic issues. Another important question to address is, whether stress might affect oscillators or clocks that exist in the brain, outside the SCN. This may be particularly important in relation to psychiatric disorders such as depression.

Indeed, brain regions outside the hypothalamus have also been reported to present daily oscillations in expression of clock genes. A study with immunohistochemistry to assess PER2 expression in rats in basal conditions, under a 12:12 LD cycle, showed that 18 forebrain

areas important for motivated and appetitive behavior displayed PER2 rhythms with brain region specific phases (Harbour et al., 2013). PER2::LUC rhythm in CA1, CA2, CA3 and DG of the hippocampus was also confirmed in isolated mouse hippocampus (Wang et al., 2009). Other areas involved in regulation of mood (cortex area 1, lateral habenula, periaqueductal grey and ventral tegmental area) were also reported to exhibit PER2::LUC circadian rhythm with more than two peaks in isolated cultures (Landgraf et al., 2016).

Two forebrain nuclei involved in fear and stress-related behavior, the bed nucleus of the stria terminalis and the central nucleus of the amygdala also present circadian PER2 expression rhythm and it is dependent on adrenal gland integrity (Amir et al., 2004). Furthermore, corticosterone replacement in adrenalectomized rats in drinking water, but not via constant-release pellets, can restore the circadian expression of PER2 in these areas. The results show the importance of corticosterone rhythm to maintain the rhythm in these nuclei. Other two stress sensitive structures, the hippocampus and cortex may also be sensitive to variations in corticosterone, since 3 days of restraint stress phase-advanced *Per1* and *Per2* expression in these areas (Tahara et al., 2015).

These studies suggest that chronic stress, by disrupting glucocorticoid rhythm, may affect brain areas important in emotional processing and consequently play a role in the development of mood disorders. Chronic high levels of glucocorticoids have been observed in depressed patients (Aihara et al. 2007, Bauer et al. 2003) and particularly for major depression, circadian desynchronization is associated with more severe symptoms, as observed in depressed patients who show desynchronization between the sleep/wake cycle and the SCN (Emens et al., 2009). Since it is very difficult to stablish the relationship of cause and consequence between stress, circadian disruption and psychiatric disorders in humans, we attempted to untangle these associations by using the social defeat model in mice. Although the defeated animals did not show depressive-like behaviors (Chapter 6), we showed that stress can cause an internal desynchronization, at least between the SCN and liver, and that glucocorticoids might be the mechanism.

5. Future prospects and conclusion

There are still many questions to be answered about the mechanisms and consequences of chronic stress on circadian rhythms.

Future studies that could follow are mentioned in item 2: inhibition of glucocorticoids by metyrapone, and perhaps by adrenalectomizing the animals, and inhibition of catecholamines production or blocking their action, to determine if those stress hormones are essential for the phase-shift observed in the liver oscillator.

Another line to further investigate are the consequences of the disruption between master and peripheral oscillators caused by stress. However, since glucocorticoids are involved not only in timing information for peripheral oscillators, but in metabolic and physiological processes, it might be difficult to differentiate the effects of rhythm disturbance

and effects caused by elevation of glucocorticoids per se.

From our studies, we can conclude that chronic social stress does not seem to affect the SCN, but it does affect the peripheral oscillator in the liver and CORT is a viable candidate for mediating this effect (Figure 1).

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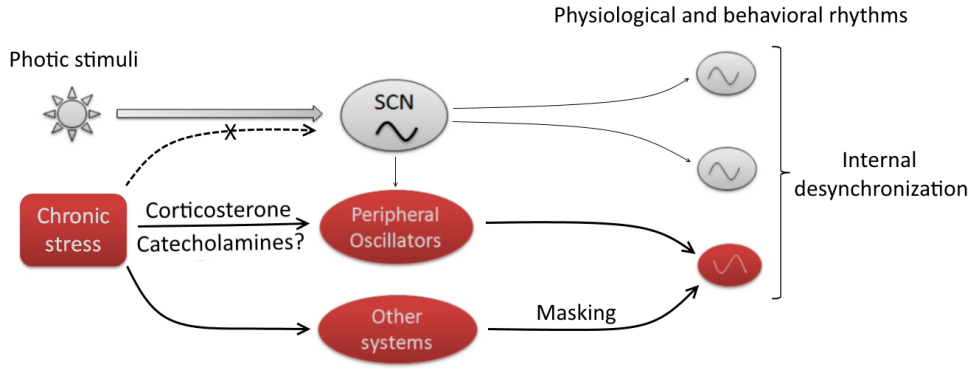

Figure 1. Conclusions on chronic stress effects on circadian rhythms. The master clock in the SCN is synchronized by the photic information, but it is not affected by stress stimuli. On the other hand, the peripheral clock in the liver is affected by chronic stress and one pathway is by corticosterone signal. Although other experiments are necessary to assess whether catecholamines can also affect the liver or other peripheral oscillators, the phase shift caused by chronic stress could result in internal desynchronization among different tissues.

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Samenvatting

Alle organismes op aarde zijn geëvolueerd in een omgeving die gekenmerkt wordt door duidelijke 24-uurs ritmes in licht-donker, temperatuur en voedselbeschikbaarheid, veroorzaakt door de draaiing van onze planeet om haar as. Als aanpassing op deze cyclische omgeving vertonen vrijwel alle soorten, inclusief de mens, dag-nacht ritmes in gedrag en fysiologie, bijvoorbeeld in slaap-waak gedrag, lichaamstemperatuur en hormoonafgifte. Deze ritmes in gedrag en fysiologie worden intern gestuurd door een systeem van biologische klokken of oscillatoren. Dit circadiane systeem (circa = ongeveer, dies = een dag) bevat een centrale klok of pacemaker die gelokaliseerd is in de suprachiasmatische nucleus (SCN) van de hypothalamus. Deze pacemaker is gelegen bovenop de kruising van de oogzenuwen en ontvangt directe licht-input vanuit de ogen, zodat het zijn eigen ritme kan synchroniseren met de 24-uurs dag-nacht ritme van de omgeving. De SCN coördineert en synchroniseert vervolgens de klokken van andere weefsels en organen zodat alle ritmes in ons lichaam op elkaar en op de omgeving zijn afgestemd en op die manier bijdragen aan een optimaal functioneren.

Als het circadiane systeem verstoord raakt en de interne 24 uurs-ritmes niet in de pas lopen met elkaar of met de omgeving, zoals vaak het geval is bij werken in nachtdiensten of bij een jetlag, dan kan dat ernstige gevolgen hebben voor het functioneren en welzijn. Op lange termijn kan een verstoring van het circadiane systeem zelfs bijdragen aan een verhoogde gevoeligheid voor allerlei ziektes en aandoeningen.

In deze context van een relatie tussen circadiane organisatie en gezondheid is het een belangrijke vraag of het circadiane systeem gevoelig is voor verstoringen veroorzaakt door stress. Omstandigheden met oncontroleerbare en chronische stress worden vaak gezien als een belangrijke oorzaak van verminderd functioneren en ziekte, en veel ziektebeelden zijn geassocieerd met verstoringen in de normale dag-nacht ritmes in gedrag en fysiologie (verstoorde slaap, verstoorde hormoonritmes). Op basis van deze gegevens zou men kunnen veronderstellen dat een verstoorde circadiane organisatie een rol speelt bij het ontstaan van stress-gerelateerde aandoeningen zoals cardiovasculaire aandoeningen en psychiatrische ziektebeelden.

In het echte leven is het moeilijk om oorzakelijke verbanden vast te stellen tussen stress, verstoorde circadiane ritmiek en verminderde gezondheid. Om deze reden is er in het onderzoek beschreven in dit proefschrift gebruik gemaakt van een model voor chronische sociale stress in muizen om de volgende vragen te beantwoorden: 1. Heeft oncontroleerbare chronische stress invloed op de centrale circadiane pacemaker in de SCN en de gedragsritmes die gecoördineerd worden door deze klok? 2. Heeft oncontroleerbare chronische stress invloed op perifere klokken elders in het lichaam en kan het de coördinatie tussen de centrale klok in de SCN en die perifere klokken verstoren?

In hoofdstuk 2 van dit proefschrift wordt een breed overzicht gegeven van de

beschikbare wetenschappelijke literatuur en momentane inzichten over effecten van stress op het circadiane systeem. Eerdere studies van onder andere onze eigen onderzoeksgroep suggereerden dat de centrale circadiane pacemaker in de SCN niet heel gevoelig lijkt te zijn voor acute stress. Echter, de literatuur leek minder consistent over de mogelijke effecten van chronische stress. Hoewel diverse studies ook na chronische stress geen veranderingen lieten zien in circadiane organisatie waren er ook enkele studies die suggereerden dat er mogelijk kleine, accumulerende effecten zijn die geleidelijk aan leiden tot een verschuiving van ritmes die gestuurd worden door de SCN. Het is bij die studies echter niet duidelijk of er daadwerkelijk sprake was van faseverschuivingen van de SCN-ritmes of dat de intern gegenereerde ritmes gemaskeerd waren door verstoringen elders in het lichaam. Daarnaast waren er enkele studies die hebben laten zien dat bepaalde hormonen die vaak geassocieerd worden met stress, met name glucocorticoïden afgegeven door de bijnieren, kunnen leiden tot verschuivingen in het ritme van perifere klokken zoals die in de lever. Deze bevindingen tezamen waren de motivatie voor het uitvoeren van een serie nieuwe experimenten in muizen waarin werd gekeken naar de invloed van chronische sociale stress op de centrale klok in de SCN en de perifere klok in de lever.

In hoofdstuk 3 onderzochten wij allereerst of chronische sociale stress van invloed is op het circadiane ritme in locomotoractiviteit waarvan bekend is dat het wordt aangestuurd door de pacemaker in de SCN. Volwassen mannelijke muizen werden gedurende het gehele experiment gehouden in constant rood licht, wat voor muizen in feite constant donker is omdat ze het rode licht nauwelijks kunnen waarnemen. Doordat de muizen onder deze constante omstandigheden waren gehuisvest, vertoonden ze een zogenaamd vrijlopend activiteitsritme met een endogene, door de klok in de SCN gegenereerde periode die iets korter was dan 24 uur. Op deze manier kon goed onderzocht worden of de fase en periode van de centrale klok beïnvloed worden door stress. De helft van de muizen blootgesteld aan chronische sociale stress door ze gedurende 10 opeenvolgende dagen kortdurend te huisvesten in het territorium van een dominante en agressieve soortgenoot, het zogenaamde ‘social defeat stress’ model. Verschillende groepen muizen werden blootgesteld aan sociale stress tijdens de circadiane activiteitsfase of tijdens de circadiane rustfase. Vervolgens werd de periode en fase van het activiteitsritme voor, tijdens en na de 10 dagen sociale stress in deze muizen vergeleken met de ritmes van controle dieren die niet aan stress werden blootgesteld. De resultaten lieten zien dat sociale stress weliswaar leidde tot een sterke reductie in de hoeveelheid activiteit, maar niet leidde tot significante veranderingen in de periode en fase van het ritme ten opzichte van de controle muizen. In overeenstemming met de meeste eerdere studies suggereert deze bevinding dat de centrale klok in de SCN niet wordt verstoord door chronische stress.

In hoofdstuk 4 werd nogmaals bevestigd dat de herhaalde sociale stress gedurende 10 dagen geen invloed had op de periode en fase van het ritme in locomotoractiviteit. Vervolgens werd er direct na de periode van sociale stress materiaal van hersenen en lever

verzameld om in vitro te kijken naar het circadiane ritme van het eiwit PERIOD2 (PER2) dat deel uitmaakt van het moleculaire klokmechanisme. Om in de verschillende weefsels het ritme in het PER2-eiwit te kunnen meten werd gebruik gemaakt van transgene PERIOD2::LUCIFERASE knock-in muizen waarin de expressie van dit klokeiwit gekoppeld is aan de expressie van het enzym luciferase. Dit enzym breekt luciferin af dat aanwezig is in het medium waarin de weefselmonsters worden gehouden en hierbij komt licht vrij dat vervolgens gemeten kan worden. Op deze manier wordt het circadiane ritme in expressie van het klokeiwit tot uiting gebracht in een meetbaar ritme in lichtproductie. Met deze methode kon direct in vitro vastgesteld worden wat het effect was van voorafgaande stress op de centrale klok in de SCN en op de perifere klok in de lever. De in vitro metingen lieten zien dat het circadiane PER2 ritme in de SCN niet beïnvloed werd door de stress, in overeenstemming met de bevinding dat de activiteits-output van de SCN niet was verschoven. Het in vitro PER2 ritme in het leverweefsel daarentegen was duidelijk verschoven in de muizen die voorafgaand gedurende 10 dagen aan sociale stress waren blootgesteld. Het PER2-ritme in de lever van deze muizen was duidelijke vertraagd ten opzichte van het PER2-leverritme in de controle muizen. Deze bevindingen ondersteunen duidelijk het idee dat de centrale klok in de SCN weliswaar goed beschermd is tegen stress maar dat sommige perifere klokken wel beïnvloed kunnen worden door stressvolle gebeurtenissen.

In hoofdstuk 5 werd onderzoek gedaan naar het mogelijke mechanisme waarlangs stress de leverklok zou kunnen verschuiven. De hypothese was dat het bijnierhormoon corticosteron een rol zou kunnen spelen. Dit hormoon wordt in sterke mate afgegeven tijdens sociale stress. Er werden opnieuw transgene PERIOD2::LUCIFERASE knock-in muizen gebruikt voor het genereren van in vitro culturen van SCN en leverweefsel. Een deel van deze culturen werd vervolgens blootgesteld aan verschillende doseringen van corticosteron. Zoals verwacht werd de periode en fase van het PER2-ritme in de SCN-culturen niet beïnvloed door directe toediening van corticosteron. Het PER2-ritme in het leverweefsel daarentegen werd duidelijk vertraagd door de toevoeging van corticosteron, in overeenstemming met de effecten van sociale stress in het vorige hoofdstuk. Tevens sluiten deze resultaten aan bij het gegeven dat receptoren voor het glucocorticoïd hormoon voorkomen in vrijwel alle weefsels in het lichaam, maar niet in de volgroeide SCN. De resultaten van dit experiment ondersteunen het idee dat het bijnierhormoon corticosteron betrokken zou kunnen zijn bij de effecten van chronisch sociale stress op perifere klokken.

De studie in hoofdstuk 6 was gebaseerd op het idee dat een interne desynchronisatie van circadiane klokken in verschillende weefsels gevolgen zou kunnen hebben voor de lichamelijke en mentale gezondheid. In deze studie werden verschillende gedragstesten uitgevoerd in muizen blootgesteld aan het sociale stress protocol en controle muizen. Deze gedragstesten waren met name gericht op het vaststellen van angst en depressie-achtig gedrag. In tegenstelling tot de verwachting werden er geen effecten van chronische sociale stress op gedrag gevonden, ondanks dat eerdere studies al duidelijke veranderingen hebben

gerapporteerd in dit type gedragingen na zowel acute als chronische sociale stress. Een mogelijke verklaring hiervoor zou kunnen zijn dat de dieren in onze studie ouder waren dan in de meeste rapportages. Jongere dieren zouden gevoeliger kunnen zijn voor de gevolgen van sociale stress. Dit neemt niet weg dat de duidelijk effecten van chronische sociale stress op de hoeveelheid locomotoractiviteit in de thuiskooi en de effecten op de leverklok gemeten in eerdere hoofdstukken blijkbaar niet direct geassocieerd zijn met parallelle veranderingen in angst en depressie-achtig gedrag.

In het laatste hoofdstuk van dit proefschrift worden alle voorgaande resultaten geïntegreerd en bediscussieerd in relatie tot ander gepubliceerd werk. Het door ons toegepaste protocol van chronische sociale stress leidde tot een duidelijke onderdrukking van locomotoractiviteit. In dat opzicht lijkt het een uiterst bruikbaar model voor onze doelstelling. De bevinding dat de periode en fase van de centrale klok in SCN niet beïnvloed wordt door deze vorm van stress is in overeenstemming met de meeste andere publicaties over de effecten van acute en chronische stress. Eén recente studie rapporteerde een verschuiving van de circadiane activiteitspiek na chronische sociale stress, maar aangezien deze studie werd uitgevoerd met muizen die waren gesynchroniseerd aan een normale 24-uurs licht-donker cyclus is het onduidelijk of er daadwerkelijk sprake was van een faseverschuiving van de endogene centrale klok. Onze bevinding dat zowel chronische sociale stress in vivo alsook chronische blootstelling aan corticosteron in vitro kan leiden tot een verschuiving van de perifere leverklok, gemeten aan de fase van het PER2-ritme in vitro stress, is eveneens in overeenstemming met diverse andere recente publicaties. Zo is er laten zien dat ook in vivo injecties met corticosteron of vergelijkbare synthetische glucocorticoïden kan leiden tot een verschuiving in het ritme van klokeiwitten in de lever en andere weefsels. Onze resultaten met toevoeging van corticosteron aan het in vitro medium suggereren dat deze effecten het gevolg kunnen zijn van directe inwerking van corticosteron op deze perifere weefsels in plaats van een indirect effect. Om de causale rol van corticosteron bij de door ons gevonden verschuiving van de leverklok na stress daadwerkelijk vast te stellen zullen toekomstige experimenten gericht zijn op het blokkeren van corticosteron afgifte tijdens de sociale stress periode. Daarnaast stellen wij voor om ook studies te verrichten naar de effecten van sociale stress op ritmes in klokeiwitten in verschillende hersengebieden buiten de SCN, met name hersengebieden die betrokken zijn bij de regulatie van cognitieve functies en emoties zoals de hippocampus en de prefrontale cortex. Dergelijke studies kunnen inzicht geven in de mogelijke rol van stress-geïnduceerde interne circadiane desynchronisatie en de gevoeligheid voor het ontwikkelen van psychiatrische aandoeningen zoals depressie.

Summary

We humans, as well as several living organisms, present biological rhythms of approximately 24 hours, such as the sleep-wake cycle, locomotor activity, temperature, and hormone secretion rhythms. The maintenance of these rhythms allows us to perform various activities in an optimal time of the day, taking advantage of the variation of light and temperature, among other environmental cycles. The 24-h light-dark cycle is caused by the Earth's rotation and influences other environmental cycles, such as temperature, humidity, availability of food, among others. Therefore, it is conceivable that organisms have evolved a temporal system that allows them to carry out certain activities at the best time of the day, according to the variation of the environmental cycles.

Several studies have shown that in mammals, this temporal system, also called the circadian (about a day) system, has a primary center in the brain that generates an internal rhythm of approximately 24 h, located in the suprachiasmatic nucleus (SCN). In addition, some other body tissues are also able to perform their functions, such as metabolism, with the same period as the rhythm produced by the SCN. This is because every cell appears to have a "molecular clock", provided by the production of some proteins in a near 24-h cycle, the so-called "clock proteins". Thus, some peripheral tissues, i.e., other than the SCN, are able to maintain the rhythm in cultures outside the body. However, the SCN produces a stronger and lasting rhythm, aligns its rhythm to the environment light-dark cycle and sends signals to synchronize the rhythms generated in other tissues. For that reason, it is also known as the central clock or oscillator.

It is important that our circadian system is synchronized with the external environment, so that we can feed, sleep and wake up, work, among other activities, at optimal times. And when this does not happen, we can see the negative effects on attention, sleep, digestion. This is what happens when we travel to places with time zones different from ours or also with shiftworkers.

Since it is important to keep our organism adjusted to external time, and the most important environmental clue for this to occur in mammals is the light-dark cycle, it is desirable that the central oscillator is not influenced by clues other than light or clues that cannot be predicted. With this knowledge in mind, we wondered if the SCN would be protected from stressful situations, which are often unpredictable and unavoidable in today's society. For humans, social stress, especially when chronic, is one of the most severe kinds of stress and is related to the development of metabolic diseases and some psychiatric disorders, such as depression. However, in our life, it is difficult to establish a cause-and-effect relationship among stress, circadian rhythm disturbance and health consequences. For this reason, we used a social stress model in mice to assess the stress effects on the circadian activity rhythm, which is controlled by the SCN and easily evaluated. We proposed to answer the following questions: 1. Is chronic social stress capable of affecting the circadian

locomotory rhythm and maintenance of the rhythm in the central clock? 2. Is chronic social stress capable of affecting the temporal organization of the central oscillator and oscillation in a peripheral tissue?

In Chapter 2, we reviewed some studies that investigated the effects of stress on circadian rhythms. Previous studies from our group have already shown that acute social stress reduces locomotor activity. However, the time when there is a sharp increase in activity and the duration (period) of the activity-rest cycle do not change. This evidence suggests that the SCN continues to maintain its rhythm and is not affected by social stress. Regarding the effects of chronic stress, the results of studies available in the literature are still contradictory. One of the reasons may be that the shape of the observed rhythms can be altered by several factors. For example, the body temperature rhythm may reflect, not only the rhythm expressed by the biological clock, but also be altered by some momentary physical activity or exposure to external heat or cold. Therefore, as discussed in the second chapter, several methodological precautions must be taken so that the observed rhythm is not masked by external or other internal factors.

In Chapter 3, we investigated whether the rhythm of locomotor activity is affected by chronic social stress. For this experiment, we kept our animals in constant red light (which is almost imperceptible to rodents and would resemble constant darkness). To measure locomotor activity, we used activity wheels. Part of the animals were exposed to an aggressive mouse (for the social stress) a few minutes, for ten days, during the active time or during the inactive time, and their rhythms were compared to animals that were not exposed to stress. Interestingly, , the stressed mice showed reduced levels of activity, but the time of the sharp increase in activity and the period of the activity rhythm did not change, similar to that observed with acute social stress in rats. These results suggest that the SCN is not affected by chronic social stress either.

In Chapter 4, we replicated the observations of the previous chapter regarding the effect of stress on the rhythm of locomotor activity. During the 10 days of social stress, the defeated animals showed lower activity levels, but the time of the sharp increase in activity and the period were not different from the non-stressed animals. In addition, we also analyzed the rhythm of expression of one clock protein in tissue cultures of SCN and liver. This analysis is important because it allows us to observe the effects of stress in these isolated areas. For this experiment, we used transgenic mice that produced this clock protein linked to a protein that produces bioluminescence, which permitted us to detect luminescence, and therefore, the rhythm of protein production. As expected, the rhythm of this clock protein production in the SCN was not different between the two groups. However, in the liver, the peak time of production of the clock protein was delayed in tissues from animals that had experienced social stress. These results support the idea that the central clock is protected against stress, but the peripheral clock in the liver is not.

In Chapter 5, we investigated a possible mechanism by which stress could affect the

clock in the liver, as observed in the experiment of the previous chapter. Our supposition was that corticosterone, a hormone that is released in greater amounts in response to stress, could be responsible for changing the rhythm of production of that clock protein in the liver. To investigate this hypothesis, we made liver and SCN tissue cultures of transgenic mice of the same type as in the previous chapter and added corticosterone in part of the cultures. The rhythm of production of the clock protein was not different in the treated from the untreated SCN cultures. On the contrary, the peak time of this protein production was delayed in liver cultures in which the hormone was added in medium concentrations. Again, the result supports the idea that the SCN is not affected by stress, but the peripheral clock in the liver is, and a possible mechanism is the increase of corticosterone in response to social stress.

Considering the idea that desynchrony between the SCN- and the peripheral clock time, such as in the liver, may have consequences for mental health, we also performed behavioral tests after the social stress protocol in mice (Chapter 6). The tests were chosen with the aim to allow us to observe if the animals display depressive-type behaviors, i.e., those resembling what is observed in depressed patients. Although some research groups had already observed these effects after social stress, we did not find differences between stressed and non-stressed animals. Perhaps the age of the animals (adults, while other groups use adolescent animals) could explain this difference, since youngsters may be more vulnerable to the effects of stress. In addition, other methodological differences in the stress protocol and behavioral assessment might explain the different results we have obtained compared to other works.

In Chapter 7, we discussed the results obtained, comparing them to other studies and their implications, in addition to suggesting future experiments. Regarding the effects of stress on the rhythm of locomotor activity, although one study observed a change in the time of peak activity, our results are in agreement with others, which did not observe changes in the rhythm. This disagreement may be related to methodological differences, such as keeping the animals in constant red light, whereas the other study, kept the animals under a light-dark cycle. Other studies have also showed that injection of corticosterone or a similar synthetic hormone also changed the peak of clock protein production, in the liver and other peripheral organs. Moreover, other hormones that are secreted in response to stress, such as adrenaline and noradrenaline, also appear to have the same effect. Therefore, we proposed a future study to block these hormones and evaluate if the effects of chronic social stress would still be the same. In addition, since other areas of the brain also appear to show rhythmic production of clock proteins, it would be interesting to study the effects of social stress in areas associated with emotional regulation, since desynchronization of biological rhythms seems to be associated with disorders such as depression. In conclusion, the central biological clock seems to be protected against the effects of chronic social stress, but the peripheral clock in the liver does not. The desynchronization between these clocks

and perhaps other peripheral clocks may be associated with health problems, such as the ones observed in shiftworkers.

Sumário

Nós humanos, assim como diversos organismos vivos, apresentamos ritmos biológicos de aproximadamente 24 h, por exemplo, o ciclo sono-vigília, ritmo de atividade locomotora, temperatura e de secreção de hormônios. A manutenção desses ritmos nos permite realizar diversas atividades no melhor horário do dia, aproveitando a variação de luz e temperatura, entre outros ciclos ambientais. O ciclo claro-escuro de 24 h é causado pela da rotação da Terra e influencia outros ciclos ambientais, como temperatura, humidade, disponibilidade de alimento, entre outros. Portanto, é concebível que os organismos tenham evoluído um sistema temporal que os permite realizar certas atividades no melhor horário do dia, de acordo com a variação dos ciclos ambientais.

Diversos estudos mostraram que nos mamíferos, esse sistema temporal, também chamado de sistema circadiano (cerca de um dia), tem um centro primário no cérebro que gera um ritmo interno de aproximadamente 24 h, localizado no núcleo supraquiasmático (NSQ). Além disso, outros tecidos do corpo também são capazes de realizar suas funções, por exemplo, metabolismo, com o mesmo período do ritmo produzido pelo NSQ. Isso porque todas as células parecem ter um "relógio molecular", provido pela produção de algumas proteínas num ciclo póximo a 24 h, as chamadas "proteínas relógio". Deste modo, alguns tecidos periféricos, isto é, outros que não o NSQ, são capazes de manter o ritmo em culturas fora do corpo. No entanto, o NSQ produz um ritmo mais robusto e duradouro, alinha seu ritmo ao ciclo claro-escuro ambiental e manda sinais para sincronizar os ritmos gerados em outros tecidos. Por essa razão, ele é também conhecido como relógio, ou oscilador, central.

É importante que nosso sistema circadiano esteja sincronizado ao horário do ambiente externo para que possamos nos alimentar, dormir e acordar, trabalhar, entre outras atividades em horários mais favoráveis. E quando isso não ocorre, podemos ver os efeitos negativos na atenção, sono, digestão. Isso ocorre, por exemplo, quando viajamos para lugares com fuso horário muito diferente do nosso ou com trabalhadores de turno.

Uma vez que é importante manter nosso organismo ajustado aos horários externos, e a pista ambiental mais importante para que isto ocorra em mamíferos é o ciclo claro-escuro, é de se desejar que o oscilador central não seja influenciado por outras pistas diferentes da luminosidade ou pistas que não sejam previsíveis. Com isto em mente, nos perguntamos se o NSQ seria protegido de situaçoes de estresse, que geralmente são imprevisíveis e muitas vezes inevitáveis na sociedade atual. Para nós humanos, o estresse social, principalmente quando crônico, é um dos mais graves e está relacionado ao desenvolvimento de doenças metabólicas e alguns distúrbios psiquiátricos, como a depressão. No entanto, no nosso dia-a-dia é difícil estabelecer uma relação de causa e consequencia entre estresse, distúrbio do ritmo circadiano e consequencias na saude. Para isso, utilizamos um modelo de estresse social em camundongos para avaliar seus efeitos no ritmo circadiano de atividade, que é controlado pelo NSQ e simples de ser avaliado. Nós propomo-nos a responder as seguintes questões: 1. O estresse social crônico é capaz de afetar o ritmo circadiano de locomoção e

manutenção do ritmo do oscilador central ? 2. O estresse social crônico é capaz de afetar a organização temporal do oscilador central e oscilação de um tecido periférico?

No capítulo 2, nós revisamos alguns estudos que investigaram os efeitos do estresse nos ritmos circadianos. Estudos prévios do nosso grupo já haviam demonstrado que o estresse social agudo reduz drasticamente os níveis de atividade locomotora em ratos. Porém, o horário em que há um aumento acentuado na atividade locomotora e a duração (período) do ciclo de atividade-descanso não são alterados. Essas evidências sugerem que o NSQ continua a manter seu ritmo e não foi afetado pelo estresse social. No entanto, os dados disponíveis na literatura sobre os efeitos do estresse crônico ainda eram contraditórios. Isso porque o formato dos ritmos observados pode ser alterado por diversos fatores. Por exemplo, o ritmo de temperatura corporal exibido pode ser não apenas resultado do ritmo expressado pelo relógio biológico, como também alterado por alguma atividade física momentânea ou exposição ao frio ou calor externo. Portanto, como discutido no segundo capítulo, diversos cuidados metodológicos devem ser tomados para que o ritmo observado não seja mascarado por fatores externos ou resultado de outros fatores internos.

No capítulo 3, nós investigamos se o ritmo de atividade locomotora é afetado pelo estresse crônico social. Para este experimento, mantivemos nossos animais em luz vermelha constante (que é quase imperceptível para roedores, o que se assemelharia ao escuro constante). Para medir a atividade locomotora, utilizamos rodas de atividade. Parte dos animais foi expostas a um camundongo agressivo (para o estresse social) durante alguns minutos, por dez dias, durante a fase de atividade ou fase de inatividade, e seus ritmos foram comparados a animais que não foram expostos ao estresse. Interessantemente, os camundongos estressados apresentaram níveis de atividade reduzida, mas o horário de início da atividade e o período do ritmo de atividade não se modificaram, de modo semelhante ao observado com estresse social agudo em ratos. Esses resultados sugerem que o NSQ também não é afetado pelo estresse social crônico.

No capítulo 4, replicamos as observações do capítulo anterior em relação ao efeito do estresse o ritmo de atividade locomotora. Durante os 10 dias de estresse social, os animais derrotados apresentaram redução nos níveis de atividade, mas o horário em que há aumento acentuado de atividade e o período não foram diferentes dos animais não estressados. Além disso, também analizamos o ritmo de expressão de uma proteína relógio em culturas de tecido do NSQ e fígado. Essa análise é importante porque nos permite observar os efeitos do estresse nessas areas isoladas. Para isso, utilizamos camundongos transgênicos que produziam essa proteína relógio ligada a uma proteína que produz bioluminescência, o que nos permitiu detectar o ritmo de produção da proteína através da luminescencia. Como esperado, o ritmo de produção dessa proteína relógio no NSQ não foi diferente entre os dois grupos. Já no fígado, o horário de pico de produção da proteína relógio aconteceu mais tarde nos tecidos provenientes de animais que passaram pelo estresse social. Esses resultados fortalecem a ideia de que o relógio central é protegido contra o estresse, mas o

relógio periférico presente no fígado não.

No capítulo 5, investigamos um possível mecanismo pelo qual o estresse possa afetar o relógio presente no fígado, como observado no experimento do capítulo anterior. Nossa suposição foi de que a costicosterona, um hormônio que é liberado em maior quantidade em resposta ao estresse, poderia ser responsável por alterar o ritmo de produção dessa proteína relógio no fígado. Para investigar essa hipótese, fizemos culturas de tecidos do fígado e do NSQ de camundongos transgênicos do mesmo tipo utilizado no capítulo anterior e adicionamos corticosterona em parte dessas culturas. Como esperado, o ritmo de produção da proteína relógio não foi diferente nas culturas de NSQ tratadas e não tratadas. No entanto, o horário do pico de produção dessa proteína foi atrasado nas culturas de fígado em que o hormônio foi adicionado em concentrações medianas. Novamente o resultado fortalece a ideia de que o NSQ não é afetado pelo estresse, mas o relógio periférico do fígado sim e um mecanismo possível é o aumento de corticosterona em resposta ao estresse social.

Considerando a ideia de que esse desalinhamento entre o horário do relógio no NSQ e de relógios periféricos, como o do fígado, possa trazer consequencias para a saúde mental, nós também realizamos testes comportamentais após o protocolo de estresse social em camundongos (Capítulo 6). Os testes utilizados foram escolhidos com o objetivo de observar se os animais apresentam comportamentos tipo-depressivos, isto é, que se assemelham a um comportamento observado em pacientes deprimidos. Apesar de alguns grupos observarem esses efeitos após os estresse social, nós não vimos diferenças entre os animais estressados e não estressados. Talvez a idade dos animais utilizados (adultos, enquanto que outros grupos utilizam animais adolescentes) possa explicar não termos observados tais efeitos, uma vez que jovens possam estar mais vulneráveis aos efeitos do estresse. Além disso, outras diferenças metodológicas no protocolo de estresse e avaliação de comportamento podem explicar os redultados diferentes que obtivemos em relação a outros trabalhos.

No capítulo 7, discutimos os resultados obtidos, comparando-os com outros trabalhos na área e suas implicações, além de sugerir experimentos futuros. Em relação aos efeitos do estresse no ritmo de atividade locomotora, embora um trabalho tenha observado mudança no horário de pico de atividade, nossos resultados vão ao encontro de outros trabalhos que não observaram mudanças no ritmo. Essa discordância pode estar relacionada a diferenças metodológicas empregadas, como manutenção dos animais em luz vermelha constante, enquanto outro estudo manteve os animais sob ciclo claro-escuro. Outros estudos também observaram que injeção de corticosterona ou de um hormônio sintético semelhante também muda o pico de produção da proteína relógio observada no fígado e em alguns outros órgãos periféricos. Além disso, outros hormônios que também são secretados em resposta ao estresse, como a adrenalina e noradrenalina também parecem ter o mesmo efeito. Por isso, propusemos um estudo futuro para bloquear esses

hormônios e observar se os efeitos do estresse social serão os mesmos. Adicionalmente, como outras áreas do cérebro também parecem apresentar produção ritmica de proteínas relógio, seria interessante estudar os efeitos do estresse social em áreas associadas à regulação emocional, tendo em vista que a dessincronização de ritmos biológicos parace estar associada a distúrbios como a depressão. Em conclusão, o relógio biológico central parece estar protegido contra os efeitos do estresse crônico social, mas o relógio periférico do fígado não. Essa dessincronização entre esses relógios e talvez outros relógios periféricos pode estar associados a problemas de saúde, como os observados em trabalhadores de turno.

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List of Publications

Ota, S.M., Suchecki, D., Meerlo, P. 2018. Chronic social defeat stress suppresses locomotor activity but does not affect the free-running circadian period of the activity rhythm in mice. Neurobiology of Sleep and Circadian Rhythms. 5: 1-7

Careaga, M.B.L. ,Tiba, P.A., Ota, S.M. Suchecki, D. 2015. Pre-test metyrapone impairs memory recall in fear conditioning tasks: lack of interaction with β-adrenergic activity. Frontiers in behavioral neuroscience. 9:51

Ota, S.M; Moreira, K.M; Suchecki, D ; Oliveira, M.G.M; Tiba, P.A. 2013. Lithium Prevents REM Sleep Deprivation-Induced Impairments on Memory Consolidation. Sleep. 36: 1677-84.

In preparation

Ota, S.M., Hut, R., Riede, S.J., Crosby, P., Suchecki, D., Meerlo, P. Social stress and glucocorticoids alter PERIOD2 rhythmicity in the liver, but not in the suprachiasmatic nucleus.

Ota, S.M., Hut, R., Suchecki, D., Meerlo, P. Effects of stress and stress hormones on endogenous clocks and circadian rhythms

Curriculum vitae

Simone Marie Ota was born on February 24th, 1987 in Santo André, Brazil. Her interest in Biology started by watching wild life videos and at the age of 12, she decided to pursue a career in this area. In 2005, she started her undergraduate studies at the Institute of Biosciences, in the University of São Paulo (USP), when she became more interested in the field of Neurobiology. She joined the group of Prof. Dr. Deborah Suchecki at 2008 to present, at the Department of Psychobiology, Federal University of São Paulo (UNIFESP), where she started her studies on stress and sleep . In 2009, she concluded her studies at USP with an essay on sleep deprivation and memory. In the following year, she started her master research on a project with the aim to prevent REM sleep deprivation effects on memory and obtained her degree in 2012. Interested in the stress effects on circadian rhythms and psychiatric disorders, she started a double-degree PhD project under supervision of Prof. Dr. Deborah Suchecki, at UNIFESP, Dr. Peter Meerlo and Prof. Dr. Roelof Hut at the Groningen Institute for Evolutionary Life Sciences, University of Groningen. The work resulting from this collaboration is presented in this thesis.

Along the years, apart from research, Simone also engaged in outreach projects, such as “Comissão de Visitas” (current “Estação Biologia”) at USP, “Curso de Verão em Psicobiologia” and “Semana do Cérebro” (Brain Awareness Week) at UNIFESP.